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Frequent downregulation of the transcription factor Foxa2 in lung cancer through epigenetic silencing

Daniela S. Basseres, PhD^{1,6,#}, Francesco D'Alò, MD, PhD^{1,#}, Beow Y. Yeap, ScD², Ester C. Löwenberg, MD, PhD¹, David A. Gonzalez, MSc¹, Hiroyuki Yasuda, MD¹, Tajhal Dayaram, PhD¹, Olivier N. Kocher, MD, PhD¹, John J. Godleski, MD³, William G. Richards, PhD³, Matthew Meyerson, MD, PhD⁴, Susumu Kobayashi, MD, PhD¹, Daniel G. Tenen, MD¹, Balazs Halmos, MD⁵, and Daniel B. Costa, MD, PhD^{1,*}

¹Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

²Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

³Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

⁴Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

⁵New York Presbyterian Hospital, Columbia University College of Physicians and Surgeons, New York, NY, USA

⁶University of São Paulo, São Paulo, SP, Brazil

Abstract

PURPOSE—We sought to determine the mechanisms of downregulation of the airway transcription factor Foxa2 in lung cancer and the expression status of Foxa2 in non-small-cell lung cancer (NSCLC).

METHODS—A series of 25 lung cancer cell lines were evaluated for Foxa2 protein expression, *FOXA2* mRNA levels, *FOXA2* mutations, *FOXA2* copy number changes and for evidence of *FOXA2* promoter hypermethylation. In addition, 32 NSCLCs were sequenced for *FOXA2* mutations and 173 primary NSCLC tumors evaluated for Foxa2 expression using an immunohistochemical assay.

RESULTS—Out of the 25 cell lines, 13 (52%) had undetectable *FOXA2* mRNA. The expression of *FOXA2* mRNA and Foxa2 protein were congruent in 19/22 cells ($p=0.001$). *FOXA2* mutations were not identified in primary NSCLCs and were infrequent in cell lines. Focal or broad chromosomal deletions involving *FOXA2* were not present. The promoter region of *FOXA2* had evidence of hypermethylation, with an inverse correlation between *FOXA2* mRNA expression and presence of CpG dinucleotide methylation ($p<0.0001$). In primary NSCLC tumor specimens, there

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*Correspondence to: Daniel B. Costa, MD, PhD - Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, 330 Brookline Av., Boston, MA 02215, Phone: 617-667-9236, Fax: 617-975-5665, dbcosta@bidmc.harvard.edu.

#equal contribution

CONFLICT OF INTEREST STATEMENT:

DBC has received consulting fees from Pfizer, Roche and AstraZeneca within the last 3 years. These conflicts did not interfere with the conduct of this study.

All other authors have no other conflict of interest to declare. None declared

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was a high frequency of either absence (42/173, 24.2%) or no/low expression (96/173, 55.4%) of Foxa2. In 130 patients with stage I NSCLC there was a trend towards decreased survival in tumors with no/low expression of Foxa2 (HR of 1.6, 95%CI 0.9–3.1; $p=0.122$).

CONCLUSIONS—Loss of expression of Foxa2 is frequent in lung cancer cell lines and NSCLCs. The main mechanism of downregulation of Foxa2 is epigenetic silencing through promoter hypermethylation. Further elucidation of the involvement of Foxa2 and other airway transcription factors in the pathogenesis of lung cancer may identify novel therapeutic targets.

Keywords

lung cancer; non-small-cell lung cancer; Foxa2; methylation; transcription factors; epigenetic; downregulation; adenocarcinoma; mutation; prognosis

INTRODUCTION

Lung cancer continues to lead cancer-related deaths in the United States and worldwide (1). Better understanding of the molecular alterations in lung cancer continues to be warranted to improve diagnostic and therapeutic decisions. The normal development of the lung requires a well-orchestrated set of ubiquitous and tissue-selective transcription factors, and the genes that interact in their associated networks (2). Over the last decade, it has become clear that some of these airway transcription factors can also be abnormally expressed in non-small-cell lung cancer (NSCLC) and may contribute to the pathogenesis of this cancer. CCAAT/enhancer binding protein alpha (C/EBP α), an essential transcription factor for late lung development, is commonly downregulated by an epigenetic mechanism in NSCLCs (3–6). The homeodomain transcription factor NKX2-1, previously known as thyroid transcription factor (TTF)-1, plays a major role in early lung development and the gene locus of *NKX2-1* is amplified in a subset of NSCLCs with adenocarcinoma histology (7–9). Sox2, a transcription factor with relatively abundant expression in ciliated cells of the perinatal lung that will eventually form the trachea and bronchi, is also amplified in a subset of NSCLCs with squamous cell carcinoma histology (10;11). It is postulated that other airway transcription factors may also contribute to the development of NSCLC.

The transcription factor forkhead box protein A2 (Foxa2), also known as hepatocyte nuclear factor 3 beta (HNF3 β), is part of the larger Forkhead box (*FOX*) gene family that is characterized by a DNA binding “winged helix domain” (12). The *FOXA2* gene is located in chromosome 20p11.21 and Foxa2 plays an essential role in the embryonic formation of the primitive streak and endoderm (13), with subsequent expression in the liver, pancreas, intestine, and lung (14). Foxa2 is essential for airway epithelial differentiation (15;16) and highly expressed in type II pneumocytes (17–19). As such, Foxa2 is a likely candidate in the pathogenesis of lung cancer. Our group identified Foxa2 as one of the critical targets upregulated by C/EBP α pathways in NSCLC cell lines (20) and observed that a large proportion of lung cancer cell lines had diminished amounts of *FOXA2*-specific mRNA and Foxa2 protein (20). Induced expression of Foxa2 in the NCI-H358 NSCLC cell line led to proliferation arrest and apoptosis (20). As so, we identified Foxa2 as an airway transcription factor that plays a role in lung cancer with a putative tumor suppressive function. However, the mechanisms of downregulation of Foxa2, as well as the clinical and prognostic significance of Foxa2 expression have never been thoroughly studied in NSCLC. In the present study, we have studied genetic and epigenetic mechanisms of downregulation of Foxa2 in lung cancer, as well as the pattern of expression of this transcription factor in early stage NSCLC. Our results establish epigenetic silencing of *FOXA2* as the main determinant of Foxa2 downregulation in lung cancer, and confirm that a large proportion of NSCLCs have low or absent levels of Foxa2.

METHODS

Cell culture

The following lung cancer cell lines were used (3;20). NSCLC derived: A427, A549, Calu-1, Calu-6, NCI-H23, NCI-H125, NCI-H292, NCI-H322, NCI-H358, NCI-H441, NCI-H460, NCI-H520, NCI-H596, NCI-H661, SKLU-1, SKMES-1, SW900, U1752. NSCLC cancer cell lines were grown in RPMI1640 supplemented with 10% fetal bovine serum. Small cell lung cancer (SCLC) derived: NCI-H60, NCI-H69, NCI-H187, NCI-H211, NCI-H345, NCI-H526, N417. SCLC cell lines were grown in RPMI1640 supplemented by HITES medium (Sigma Chemical Co., St. Louis, MO). DNA, RNA and protein extracts were isolated as described previously (3;20;21).

Western and northern blotting

Protein whole-cell lysates were prepared as reported previously (20;22). A 1:1000 dilution of a polyclonal goat anti-Foxa2 antibody (under the name HNF3 β from Santa Cruz Biotechnology, Santa Cruz, CA), and a 1:10000 dilution of a monoclonal mouse anti- β -actin (Sigma Life Science, St. Louis, MO) were used. Total cellular RNA from cell lines was isolated and probed as detailed previously (20).

DNA sequencing, bisulfite sequencing and copy number analysis

The promoter region and three exons of *FOXA2* were sequenced by the use of seven primer sets as described previously (20), and compared with that of wild-type (WT) *FOXA2* (Genbank AF176110, under the name HNF3 β). Bisulfite sequencing was performed as described previously (20). The following primers were used: sense, 5'-TTGGAAGATAGAGAGGATAGA-3'; and antisense, 5'-CCCCCTCCCTATTACCAATTCAA-3'. Peripheral blood mononuclear cell DNA served as negative control, whereas universally methylated DNA (CpGenome Universally Methylated DNA; Intergen, New York, NY) was used as positive control. DNA copy number alterations were analyzed using methods described previously (7) using the Broad-Novartis Cancer Cell Line Encyclopedia. In brief, DNA was probed onto Affymetrix SNP6.0 arrays (Affymetrix Inc., Santa Clara, CA). The converted and normalized data was then visualized using the Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA) and "Foxa2" as a search term. Heatmap default reads were in the range of -1.5 to 1.5, with values below or above considered potential evidence of segment deletion or amplification, respectively.

Deoxyazacytidine treatment and real time PCR for *FOXA2*

5'-aza-2'-deoxycytidine (deoxyazacytidine) was obtained as described previously (20). Cell lines (NCI-H23 and SKLU-1) were treated with 1 μ M of deoxyazacytidine and RNA collected. The *FOXA2* and 18S rRNA primers and conditions used have been previously described (20).

Tumor sample acquisition

For the initial immunohistochemical studies, NSCLC tumor samples were identified through the Beth Israel Deaconess Medical Center database and paraffin-embedded tissue specimens obtained. For the confirmatory immunohistochemical study, stage I NSCLC tumor samples from a prospective study performed from 1984 to 1992 at Brigham and Women's Hospital and Dana-Farber Cancer Institute (23) were used. The original 244 patients of this cohort consisted of 108 women and 136 men with mean age of 64 years, and adenocarcinomas comprising 58% of tumors. Most patients underwent a curative lobectomy (71%), while segmental resections (23%) and pneumonectomy (6%) were the other predominant surgical techniques (23). The median follow-up for living patients was 65 months (23). Additionally,

isolated genomic DNA from NSCLC tumor specimens was provided by the Dana-Farber/Harvard Cancer Center database for sequencing analysis. These DNA samples had been previously sequenced for *CEBPA* mutations (24). The use of tissue material for these studies was approved by the Institutional Review Boards of Beth Israel Deaconess Medical Center and Dana-Farber Cancer Institute.

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue specimens using citrate-microwave antigen retrieval. A dilution (1:500) of a polyclonal goat Foxa2 antibody (200 µg/0.1 ml; under the name HNF3β from Santa Cruz Biotechnology, Santa Cruz, CA) was used. Specificity of staining was confirmed by the concomitant use of a specific Foxa2 blocking peptide and non-specific blocking peptides to C/EBPα and Ki-67 (all from Santa Cruz Biotechnology, Santa Cruz, CA). Results with these blocking peptides (data not shown) confirmed the specificity of the antibody for Foxa2 in NSCLCs. Immunohistochemistry was performed using Vectastain ABC kits (Vector Laboratories, Burlingame, CA). Positive staining was visualized by incubating the slides with diaminobenzadine. Tumor blocks were chosen to contain areas of viable tumor as well as some adjacent normal lung. Scoring of specimens was blindly performed comparing tumor staining to the staining of basal bronchial cells or type 2 pneumocytes, which were scored as 3+. Individual samples had homogeneous Foxa2 nuclear staining in tumor cells. Samples were scored semi-quantitatively according to the following criteria: lack of nuclear staining (score 0), weak staining (score 1+), moderate staining (score 2+) and intense staining similar to the bronchial cell internal control (score 3+).

Statistical analysis

Comparison of promoter hypermethylation or clinicopathologic characteristics and Foxa2 expression was based on Cohen's Kappa and Fisher exact tests. Overall survival (OS) was calculated from time of surgery to time of death of any cause or to time of last follow-up, at which point the data were censored, as described previously (23). The Kaplan-Meier method was used to estimate OS, while the survival difference between patient groups was assessed by the hazard ratio (HR) estimated using the proportional hazards model. All reported p-values are based on a two-sided hypothesis. The data analysis was conducted primarily using SAS 9.1 (SAS Institute Inc, Cary, NC).

RESULTS

Mechanisms of downregulation of Foxa2 in lung cancer cell lines and mutational analysis of *FOXA2* in NSCLC

Using a panel of 18 NSCLC derived and 7 SCLC derived cell lines, we observed that 13 out of 25 (52%) lines had undetectable mRNA levels of *FOXA2* by northern blot (Table 1). Protein levels of Foxa2, by western blot, were undetectable in 8 out of 22 (36.3%) lines analyzed (Table 1). In these 22 cell lines, we observed a statistically significant correlation between *FOXA2* mRNA and protein levels ($p=0.001$, Table 1). Figure 1A shows representative western blot results for 6 of these lines, 3 with (A549, NCI-H358, NCI-H441) and 3 without (A427, NCI-H23, SKLU-1) expression of Foxa2 protein.

We sequenced the three exons of *FOXA2* in DNA isolated from 32 primary NSCLC tumor samples: 19 adenocarcinomas and 13 squamous cell carcinomas. No mutations (0 out of 32, 0%) were identified. Out of these 25 cell lines, sequence of the *FOXA2* gene (Table 1) had previously disclosed 2 mutations (20). To evaluate copy number changes of the chromosomal region 20p11.21, we were able to obtain copy number data from Affymetrix SNP6.0 arrays in 17 of our 25 cell lines (Broad-Novartis Cancer Cell Line Encyclopedia).

None (0 out of 17, 0%) of these had Heatmap reads consistent with either deletion or amplification of the region flanking or at *FOXA2*'s chromosomal location (Table 1).

To determine if epigenetic changes were responsible for the downregulation of Foxa2, we analyzed bisulfite treated DNA from the 25 lung cancer cell lines and sequenced a CpG dinucleotide-rich 369 base pair area involving the promoter and 5' untranslated region of *FOXA2* (Figure 1B). 25 CpG dinucleotides encompass this segment. Each sample had at least 2 clones sequenced, with an average of 6.4 clones per sample with a range of 2 to 13 (data not shown). Figure 1C shows representative sequencing results for bisulfite treated DNA.

Out of the 13 cell lines without *FOXA2* mRNA, a total of 20.8% (485 out of 2325 sequenced) CpG dinucleotides were methylated; whereas in the 12 lines with *FOXA2* mRNA, only a total of 10% (168 out of 1675 sequenced) CpG dinucleotides were methylated ($p < 0.0001$). Out of the 13 cells with absence of *FOXA2* mRNA (Table 1), 10 had more than 10% of CpG dinucleotides methylated; whereas out of the 12 cells with presence of *FOXA2* mRNA, only 2 had more than 10% of CpG dinucleotides methylated ($p = 0.005$). It is unclear if the density of methylation reported (Table 1) reflects the true percentage of *FOXA2* promoter hypermethylation throughout the whole CpG island of *FOXA2* or only the density of methylation observed in the sequence nucleotides.

We had previously demonstrated that the demethylating agent deoxyazacytidine was able to increase *FOXA2* RNA levels in 3 cell lines (A427, NCI-H322 and NCI-H596) with baseline downregulation of Foxa2 expression (20). In the current study, treatment of NCI-H23 cells with 1 μ M of deoxyazacytidine increased detection of *FOXA2* by 9.5 fold using our real time PCR assay (from $0.03975\% \pm 0.00676\%$ at baseline to $0.38007\% \pm 0.07910\%$ of 18S rRNA $\times 100$; $n = 2$). *FOXA2* levels of cell line SKLU-1 were nearly undetectable in the presence or absence of 1 μ M of deoxyazacytidine (data not shown).

The results present here exclude *FOXA2* mutations or copy numbers changes as main determinants of the downregulation of Foxa2 RNA and protein levels in our lung cancer dataset, and highly suggest that epigenetic changes are correlated with downregulation of Foxa2.

Downregulation of Foxa2 in primary NSCLC tumors

To determine the expression pattern of Foxa2 protein levels using our four point scoring system (Methods, Figure 2), we initially analyzed 43 NSCLC tumors without clinical or outcome annotation. The main immunohistochemical staining pattern observed for Foxa2 was that of nuclear staining (Figure 2). Bronchial epithelial cells or type 2 pneumocytes, which express high levels of Foxa2 (score of 3+), were used as internal controls when available for each specimen (Figure 2). Out of the 20 adenocarcinomas, 3 had 0, 6 had 1+, 7 had 2+, and 4 had 3+ scores. Out of the 16 squamous cell carcinomas, 2 had 0, 11 had 1+, 2 had 2+, and 1 had 3+ scores. Out of the 7 with NSCLC not otherwise specified, 4 had 0, 1 had 1+ and 2 had 2+ scores. In aggregate, 9 out of 43 (20.9%) had no expression (score of 0) of Foxa2 and 27 out of 43 (62.8%) had either no or low expression of Foxa2 (scores of 0 or 1+).

To confirm these results, we used a previously described collection of stage I NSCLCs (23) with annotated pathological, clinical, molecular and survival data. We were able to perform the aforementioned Foxa2 immunohistochemistry in 130 stage I adenocarcinomas (Table 2). 33 (25.3%) had a score of 0, 36 (27.6%) had a score of 1+, 15 (11.5%) had a score of 2+, and 46 (35.3%) had a score of 3+. In these 130 NSCLCs, there was no statistically significant difference between the groups with absence (score 0) or presence (scores 1+, 2+

and 3+) of Foxa2 in regards to degree of tumor differentiation, tumor size, presence of *KRAS* mutation, or expression of p53 by immunohistochemistry (Table 2). When we looked at clinical outcomes in the evaluable 129 patients (42 deaths, with 5-year survival of 72% and median OS of 8.2 years), the survival difference between the group with Foxa2 immunohistochemistry scores of 0 (13/33 deaths, 5-year survival 64%, median OS 7.1 years) and 1+ (14/36 deaths, 5-year survival 71%, OS 6.4 years) and the group with scores of 2+ (3/15 deaths, 5-year survival 80%, OS not reached) and 3+ (12/45 deaths, 5-year survival 73%, OS not reached) corresponds to a HR of 1.6 (95% CI 0.9–3.1; $p=0.122$), a trend favoring survival in the latter group of patients with expression of Foxa2 (Figure 3).

These results support our cell line observation and confirm a high degree of either absence (42 out of 173 total NSCLCs tested, 24.2%) or no/low expression (96 out of 173 total NSCLCs tested, 55.4%) of Foxa2 in primary NSCLCs.

DISCUSSION

C/EBP α , NKX2-1, Sox2 and Foxa2 are airway transcription factors that participate in the development of the normal bronchus and lung epithelium. They have been shown to be susceptible to molecular changes that occur during lung carcinogenesis (3;6;7;7–10;10;11;20).

We have performed a comprehensive genetic characterization of the *FOXA2* gene and its chromosomal locus in lung cancer. *FOXA2* mutations were not present in primary tumor samples. The chromosomal segment 20p11.21 that contains *FOXA2* was not a site of major focal or broad deletions or amplifications in 17 cell lines analyzed. These results make it unlikely that either mutations or deletions account for the frequent loss of Foxa2 expression in lung cancer cell lines. A detailed analysis of the promoter region of *FOXA2* - which has a CpG-rich island - for epigenetic changes proved to be more informative. Evidence of hypermethylation of the *FOXA2* promoter was a frequent event in the cell lines studied (12 out of 25, 48%, had more than 10% of CpG dinucleotide hypermethylation). There was an inverse correlation between the absence of *FOXA2* mRNA or Foxa2 protein and the degree of promoter hypermethylation. We observed that treatment with the demethylating agent deoxyazacytidine was able to induce *FOXA2* mRNA in 4 cell lines (A427, NC-H23, NCI-H322 and NCI-H596) that did not express Foxa2 at baseline (20). A high-resolution mapping of DNA hypermethylation in NSCLC disclosed *FOXA2* as one of the genes that involves a CpG island that is commonly hypermethylated in early stage NSCLCs (25). In that study, 1 out of 5 stage I NSCLC samples analyzed contained promoter hypermethylation as identified by 3 probes that spanned the *FOXA2* CpG island and an additional probe was present in 4 out of the 5 samples (25). The latter data, in association with ours, add support to our premise that epigenetic changes are the likely mechanism of loss of Foxa2 expression in NSCLC. Additional studies to determine the levels of *FOXA2* promoter hypermethylation in NSCLC primary tumors are warranted to verify the correlation of the level of Foxa2 downregulation with epigenetic changes.

We were also able to demonstrate that the loss of expression of Foxa2 is a common event in primary NSCLC samples. Almost one quarter (24.2%, 42 out of 173) of the NSCLCs tissue specimens tested lacked expression of Foxa2. Weak expression or loss of expression of Foxa2 was seen in over half of the samples analyzed (55.4%, 96 out of 173). The frequent loss of Foxa2 expression when compared to the ubiquitous expression of this transcription factor in normal bronchial epithelial cells and type 2 pneumocytes further highlights that downregulation of Foxa2 is common in NSCLCs.

The prognostic significance of aberrant airway transcription factors in NSCLC is uncertain. The overexpression of NKX2-1 (TTF-1) was associated with improved prognosis when compared to the lack of expression of this protein in NSCLC, whereas the amplification of *NKX2-1* led to a worse overall prognosis (8). Both the overexpression of Sox2 and the amplification of *SOX2* were associated with improved prognosis in NSCLCs with squamous cell histology (11), whereas the expression of Sox2 in adenocarcinomas was a poor prognostic marker in early stage NSCLC (26). The downregulation of C/EBP α was not prognostic in our group's study of stage II and III NSCLC (4). In the present cohort of 130 NSCLCs, we observed a trend towards improved prognosis in the subset of stage I adenocarcinomas with higher expression of Foxa2 when compared to the group with low or no expression of Foxa2. Although our sample size was relatively small, this observation is intriguing and merits further evaluation in larger series of early stage NSCLC. It is possible to speculate that, either alone or in combination, the aberrant genetic or epigenetic patterns of the airway transcription factors C/EBP α , NKX2-1, Sox2, Foxa2 among others may have either prognostic or predictive impact in managing NSCLCs. Our group is planning to start a comprehensive analysis of the expression patterns and molecular abnormalities of the aforementioned airway transcription factors in early stage NSCLCs with a goal to correlate these among themselves and with clinical outcomes and previously established prognostic and molecular markers in NSCLC (such as age, tumor size and oncogene mutations).

Airway transcription factors have been exploited in preclinical models as therapies for NSCLC. The inhibition of NKX2-1 and Sox2 in adenocarcinomas and squamous cell carcinoma cell lines, respectively, that have amplification of these transcription factors led to inhibition of tumor growth (7;9;10); suggesting that inhibitors of these proteins may have anti-cancer effects. The induced expression of C/EBP α or Foxa2 in NSCLC cell lines that have downregulation of these transcription factors caused growth reduction, proliferation arrest and increased apoptosis; supporting C/EBP α and Foxa2 as having tumor suppressive potential in some NSCLCs (3;20). Foxa2 has also been shown to function as a suppressor of tumor metastasis in lung cancers (27). Therefore, reestablishing C/EBP α and Foxa2 mediated pathways in NSCLC may herald novel therapeutic targets. One of the target genes of the Foxa2 and C/EBP α pathways is the principal enzyme involved in prostaglandin catabolism, NAD⁺-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (28). 15-PGDH is the major prostaglandin degradation enzyme counteracting the effect of cyclooxygenase isoenzyme 2 (COX-2), and we have begun to exploit the modulation of 15-PGDH activity as a future therapeutic target in NSCLCs (28).

In summary, we have confirmed that Foxa2 is frequently downregulated in lung cancer as a result of epigenetic silencing. Foxa2 and other airway transcription factors (NKX2-1, Sox2 and C/EBP α) should be evaluated as novel therapeutic targets in NSCLC.

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Figure 1.

Epigenetic downregulation of Foxa2 in lung cancer. A) Western blot with protein expression level of Foxa2 in NSCLC cell lines; B) Schematic diagram of the promoter and exon 1 (5' untranslated region) of the *FOXA2* gene. Dash bars demonstrate sites of CpG dinucleotide areas; C) Diagrams of methylated CpG nucleotides of the CpG rich area of *FOXA2* from bisulfite treated DNA of a 369 base pair region of *FOXA2*. Five separate representative subclones are displayed for each cell line, with one row representing the 25 CpG dinucleotides present in the 369 base pair region. If CpG dinucleotides sequenced had not been modified by bisulfite (lack of cytosine [C] to uracil [U] change upon bisulfite treatment), we considered this evidence of methylation in that CpG. Dark circles indicate methylated CpG dinucleotides and white circles indicate non-methylated CpG dinucleotides. PBMC, peripheral blood mononuclear cells.

Figure 2.

Expression of Foxa2 in NSCLC using an immunohistochemical (IHC) scoring system. Internal controls for expression of Foxa2 included A) bronchial epithelial cells and B) type 2 pneumocytes, which had a score of 3+; C) 3+ IHC score (intense nuclear staining) in a tumor sample; D) 2+ IHC score (moderate nuclear staining) in a tumor sample; E) 1+ IHC score (weak nuclear staining) in a tumor sample; F) 0 IHC score (lack of staining) in a tumor sample. Black arrows indicate tumor tissue. Panels A, C and D, 100X; panels B, E and F, 400X.

Figure 3. Kaplan-Meier survival curve in 130 stage I adenocarcinomas by Foxa2 immunohistochemical (IHC) score. Patients were separated in two groups: Foxa2 IHC scores 0–1+ and Foxa2 IHC scores 2–3+.

Table 1

Foxa2 protein expression, *FOXA2* RNA expression, *FOXA2* methylation status, *FOXA2* copy number and *FOXA2* gene sequence in 25 lung cancer cell lines

Cell line	Foxa2 protein	<i>FOXA2</i> RNA	<i>FOXA2</i> CpG dinucleotide methylation	20p11.21(<i>FOXA2</i>) deletion (Heatmap read [#])	<i>FOXA2</i> DNA gene sequence
Non-small-cell lung cancer					
A427	–	–	30.6%	ND	WT
A549	+1	+1	0.5%	– (0.40)	WT
Calu-1	+1	+2	0.0%	– (0.44)	WT
Calu-6	+3	+3	0.0%	– (–0.39)	WT
NCI-H23	–	–	18.0%	– (–0.07)	WT
NCI-H125	–	–	1.6%	ND	WT
NCI-H292	+1	+1	30.2%	ND	WT
NCI-H322	–	–	22.5%	– (0.02)	WT
NCI-H358	+2	+3	9.1%	– (0.85)	WT
NCI-H441	+3	+3	6.6%	– (–0.54)	WT
NCI-H460	+1	–	21.3%	– (0.46)	WT
NCI-H520	+3	+3	0.0%	– (–0.99)	WT
NCI-H596	+1	–	13.3%	– (–0.35)	WT
NCI-H661	–	–	0.8%	– (–0.08)	WT
SKMES-1	+2	+2	0.0%	– (–0.39)	WT
SKLU-1	–	–	60.0%	– (0.01)	C deletion @ 3220 (A194fsX218)
SW900	+3	+3	0.0%	– (0.18)	WT
U1752	+1	–	3.1%	ND	WT
Small cell lung cancer					
NCI-H60	–	–	22.6%	ND	G to A @ 2916 (G92D)
NCI-H69	+2	+1	30.8%	– (0.19)	WT
NCI-H187	+1	+1	5.6%	ND	WT

Cell line	Foxa2 protein	FOXA2 RNA	FOXA2 CpG dinucleotide methylation	20p11.21(FOXA2) deletion (Heatmap read [#])	FOXA2 DNA gene sequence
NCI-H211	–	–	16.0%	– (–0.05)	WT
NCI-H345	ND	–	42.0%	ND	WT
NCI-H526	ND	+ ²	5.6%	– (0.39)	WT
N417	ND	–	20.0%	ND	WT
Controls					
PBMC	NA	NA	0.0%	NA	NA
methylated DNA	NA	NA	96.0%	NA	NA

–, absent; +, present;
¹, weak expression;
², moderate expression;
³, strong expression;
[#] default range –1.5 to 1.5;
ND, not done; NA, not applicable; PBMC, peripheral blood mononuclear cell; WT, wild-type

Table 2

Pathological and molecular characteristics of 130 stage I adenocarcinomas stained for Foxa2 using immunohistochemistry

	Foxa2 score 0 (n=33)	Foxa2 score 1+ (n=36)	Foxa2 score 2+ (n=15)	Foxa2 score 3+ (n=46)	p-value (0 and 1+ vs 2+ and 3+)	p-value (0 vs 1+, 2+, 3+)
Histology						
Adenocarcinoma	33	36	15	46		
Other	0	0	0	0	-	-
Differentiation						
Well	4	8	4	8		
Moderate	19	15	7	27	0.547	0.593
Poor	10	13	4	11		
T (size)						
T1	26	24	12	39		
T2	7	12	3	7	0.144	1.000
KRAS genotype (n=128)						
WT	20	25	8	28		
mutation	13	10	7	17	0.581	0.834
p53 IHC						
0	21	18	9	18		
1+	2	4	1	5	0.460	0.436
2+	5	7	3	8		
3+	5	7	2	15		