Overexpression of Oncoprotein 18 Correlates with Poor Differentiation in Lung Adenocarcinomas*

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We examined the expression of oncoprotein 18 (Op18) in 93 lung adenocarcinomas and 10 uninvolved lung samples using quantitative two-dimensional PAGE analysis with confirmation by mass spectrometry and two-dimensional Western blot analysis. mRNA expression was examined using oligonucleotide microarrays, and the cellular localization of the Op18 protein was examined using immunohistochemical analysis of tissue microarrays. Three phosphorylated forms and one unphosphorylated form of the Op18 protein were identified and found to be overexpressed in lung adenocarcinomas as compared with normal lung. The percentage of phosphorylated to total Op18 protein isoforms increased from 3.2% in normal lung to 7.9% in lung tumors. Both the phosphorylated and unphosphorylated Op18 proteins were significantly increased in poorly differentiated tumors as compared with moderately or well differentiated lung adenocarcinomas (p < 0.03), suggesting that up-regulated expression of Op18 reflects a poor differentiation status and higher cell proliferation rates. This was further verified in A549 and SKLU1 lung adenocarcinoma cell lines by examining Op18 levels and phosphorylation status following treatment that altered either cell proliferation or differentiation. The increased expression of Op18 protein was significantly correlated with its mRNA level indicating that increased transcription likely underlies elevated expression of Op18. The overexpression of Op18 may offer a new target for drug- or gene-directed therapy and may have potential utility as a tumor marker. Molecular & Cellular Proteomics 2:107–116, 2003.

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Oncoprotein 18 (Op18) is a ubiquitous cytosolic protein that has been independently studied in various cellular systems under different names including stathmin (1, 2), metablin (3), phosphoprotein 19 (4), and LAP18 (5). Op18 is proposed to act as a relay for a diverse array of cell signaling pathways, which influence cell growth and differentiation (6). More recently Op18 has been identified as one of the key regulators of cell division through its influence on microtubule dynamics (7). The unphosphorylated form of Op18, which predominates in the interphase portion of the cell cycle, promotes the depolymerization of microtubules by increasing the catastrophe frequency at pH 7.5 or by sequestering tubulin at pH 6.8 (8). Phosphorylated Op18, which increases during mitosis, results in a reduced affinity of Op18 for tubulin and increased microtubule stabilization, thus allowing the mitotic spindle to form (9). Op18 is variably phosphorylated on four distinct serine residues (Ser-16, Ser-25, Ser-38, and Ser-63) in intact cells by different kinases during the cell cycle (10). The incidence of lung adenocarcinoma is increasing among both men and women in the United States (11); however, the expression status of Op18 protein and its specific isoforms or its mRNA have not been examined. Given its role as an important regulator in cell proliferation and differentiation, deregulated expression of Op18 may be relevant to cancer. Overexpression of Op18 has been observed in acute leukemia (12), lymphomas (13), neuroblastoma (14), prostatic adenocarcinoma (15), ovarian cancer (16), and breast cancer (17, 18). In the present study, Op18 protein and mRNA expression were analyzed in lung adenocarcinomas and uninvolved lung samples using 2D PAGE. Quantitative measures for individual protein spots were obtained, and tandem mass spectrometry and 2D Western blot analysis were used to confirm the identity of the specific Op18 isoforms. Associations between Op18 protein and mRNA expression as well as associations with specific clinical-pathological features of the tumors were determined.
EXPERIMENTAL PROCEDURES

Tissues—Patients undergoing resection for lung cancer at the University of Michigan Hospital from May 1991 to July 2000 were evaluated for inclusion in this study. Patient identifiers were coded to protect confidentiality. Patient consent was received, and the protocol was approved by the Institutional Review Board (Medicine). Lung tumors and adjacent normal lung tissue were obtained at the time of surgery and transported to the laboratory in Dulbecco’s modified Eagle’s medium (Invitrogen) on ice. Medical records were available for each patient. A total of 64 stage I lung adenocarcinomas, 29 stage III lung adenocarcinomas, and 10 uninvolved lung tissues from these patients were examined. A portion of each tumor and/or lung tissue was embedded in optimum cutting temperature medium (OCT) (Miles Scientific, Naperville, IL), frozen in isopentane cooled with liquid nitrogen for cryostat sectioning, and then stored at −80 °C. Hematoxylin-stained cryostat sections (5 μm), prepared from tumor pieces to be utilized for protein or mRNA isolation, were evaluated by a study pathologist (T. J. Giordano) and compared with hematoxylin and eosin-stained sections made from paraffin blocks of the same tumors. Two contiguous 2-mm³ samples were removed for RNA and protein isolation, respectively. Specimens were excluded based on unclear or mixed histology (e.g. adenosquamous), tumor cellularity less than 70%, potential metastatic origin as indicated by previous tumor history, extensive lymphocytic infiltration or fibrosis, or prior chemotherapy or radiotherapy. Tumors were histopathologically divided as bronchial derived if they exhibited invasive features with architectural destruction or bronchioloalveolar if normal lung architecture was preserved.

2D PAGE, Protein Quantification, and 2D Western Blotting—Samples were solubilized in standard lysis buffer. Sample volumes of between 15 and 30 μl were immediately applied to isoelectric focusing gels containing 50 μl of ampholytes/ml. Analytical 2D PAGE, protein quantification, and 2D Western blotting were performed as described previously (19–21). Anti-stathmin/Op18 rabbit monoclonal antibody (0.6 μg/ml, Calbiochem-Novabiochem) was incubated for 1 h at room temperature.

Mass Spectrometry—Protein spots identified for analyses were removed from preparative 2D gels using extracts from A549 lung adenocarcinoma cells and lung adenocarcinoma tissues. The conditions were identical to the analytical 2D gels except that there was 30% greater protein loading and gels were silver-stained by successive incubations in 0.02% sodium thiosulfate for 2 min, 0.1% silver nitrate for 40 min, and then 0.014% formaldehyde plus 2% sodium carbonate for 10 min. Identification of proteins of interest was performed using nanoflow capillary liquid chromatography of the tryptic digests coupled with electrospray tandem mass spectrometry (MS/MS) in a Q-TOF micro (Micromass, Manchester, UK). MS/MS spectra produced by electrospray MS/MS were automatically processed and searched against a non-redundant database using ProteinLynx Global SERVER (www.micromass.co.uk).

Immunohistochemistry of Tumor Tissue Microarrays—Tissue microarray blocks were constructed according to the method of Kononen (22) from formalin-fixed, paraffin-embedded tissue blocks representing the best morphological areas of the 93 pulmonary tumors in addition to representative normal tissue. Deparaffinized sections of the tissue microarrays were microwave pretreated in citric acid to retrieve antigenicity. The sections were incubated with 1% bovine serum albumin containing blocking solution for 60 min at room temperature. After incubation with anti-stathmin (Op18) antibody overnight at 4 °C, the immunocomplex was visualized by the immunoglobulin enzyme bridge technique using Vector ABC-peroxidase kit (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine tetra-chloride as a substrate. The sections were lightly counterstained with hematoxylin.

**Fig. 1.** A, digital image of a silver-stained 2D PAGE separation of a lung adenocarcinoma showing the region containing Op18 protein spots separated by molecular mass (MW) and isoelectric point (pI). Spot Op18 represents the unphosphorylated form, and the other three spots (Op18a, Op18b, and Op18c) represent the phosphorylated Op18 isoforms. B and C, plots showing the correlation between the unphosphorylated isoforms Op18 and phosphorylated isoforms Op18a and Op18b. The expression of the unphosphorylated Op18 was strongly correlated with both phosphorylated forms Op18a and Op18b (r = 0.49 and 0.56, respectively; p < 0.01) using Spearman correlation coefficient analysis.
Affymetrix Oligonucleotide Microarrays—Total RNA was isolated from 86 of the tumors and the 10 normal lung samples used in this study using Trizol reagent (Invitrogen) as described previously (23). All protocols used for mRNA reverse transcriptase, production of cDNA, cRNA amplification, hybridization, and washing conditions for the 6800 gene HuGeneFL oligonucleotide chips are as provided by the manufacturer (Affymetrix, Santa Clara, CA). The chips were scanned using the GeneArray scanner with data analysis performed using GeneChip 4.0 software (Affymetrix). Details of data trimming and normalization are described elsewhere (23, 24).

A549 and SKLU1 Cell Culture and IL-6, IFN-γ/H9251, and Dexamethasone Treatment—A549 and SKLU1 cell lines were obtained from the American Tissue Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum until 80% confluent. After 12 h of starvation in Dulbecco’s modified Eagle’s medium without fetal bovine serum, cells were treated with each of the following treatments: dexamethasone (DX) (0.25 μM, Sigma), IL-6 (1.6 μg/ml, BIOSOURCE, Camarillo, CA) + DX, IFN-γ (0.28 μg/ml, BIOSOURCE) + DX, or 10% fetal bovine serum. Cells were treated for 24, 48, and 72 h and then washed three times with phosphate-buffered saline prior to protein isolation and subsequent analysis using 2D PAGE.

Statistical Analysis—F tests were used for testing the difference in each clinical pathology group except the tumor-normal group for which t tests were used. The relationships between the levels of Op18 protein and mRNA expression and between Op18 protein/mRNA and the protein/mRNA levels of other genes were examined using the Spearman correlation coefficient statistical method. A probability (p) level of <0.05 (two-sided) was considered statistically significant.

**TABLE I**
Level and frequency of Op18 proteins and mRNA expression in normal lung and adenocarcinomas

| Op18 isoform | Normal X ± S.D. | Tumor X ± S.D. | p (t test) | -Fold change tumor/normal | Frequency[b] in normal | Frequency[b] in tumor |
|--------------|----------------|----------------|-----------|--------------------------|------------------------|-----------------------|
| Op18         | 0.2034 ± 0.069 | 1.081 ± 0.976  | <0.0001   | 5.3                      | 3.3                    | 89.2                  |
| Op18a        | 0.0037 ± 0.009 | 0.031 ± 0.055  | <0.0001   | 8.2                      | 0.0                    | 37.6                  |
| Op18b        | 0.0017 ± 0.005 | 0.074 ± 0.126  | <0.0001   | 44.0                     | 10.0                   | 52.7                  |
| (a + b)/total| 0.0317 ± 0.056 | 0.079 ± 0.1    | 0.0320    | 2.5                      | 1.4                    | 47.7                  |
| Op18 mRNA    | 515.4 ± 70.2    | 746.7 ± 331.6  | <0.0001   | 1.4                      | 10.0                   | 47.7                  |

[a] X ± S.D., mean value ± S.D.  
[b] Cut-off value is mean value of normal lung ± 2 S.D.
false discovery rate was determined similar to the method described in Ref. 25, which uses a permutation technique to determine significance of chance in gene expression.

RESULTS

Three Phosphorylated and One Unphosphorylated Op18 Isoforms Are Expressed in Lung Adenocarcinomas—A silver-stained 2D polyacrylamide gel showing the region containing the Op18 polypeptides in a lung adenocarcinoma is shown in Fig. 1A. Spots corresponding to Op18, Op18a, and Op18b were identified as different isoforms of oncprotein 18 by mass spectrometry (Fig. 2) and 2D Western analysis (19, 30). Op18 has been previously identified as the unphosphorylated form, and Op18a and Op18b have been identified as representing phosphorylated forms of the Op18 protein (12, 26–29). 2D Western blot analysis also identified an additional isoform, Op18c, of similar molecular weight but more acidic and likely to be a triphosphorylated form in the lung tumors (19, 30). This isoform could not be quantitatively analyzed in all samples due to its very low abundance (accounting for <1% of all Op18 protein). In 80% of the tumors and in all normal samples, this form could not be detected on the 2D gels. The relative expression values of isoforms Op18, Op18a, and Op18b from the quantitative analysis of 2D gels among all 93 lung adenocarcinoma samples is 1.081, 0.031, and 0.074, respectively (accounting for 91.2, 2.5, and 5.4% of total Op18 protein abundance, respectively). The expression level of the unphosphorylated form of Op18 was much higher than either phosphorylated isoform Op18a (34.9-fold) or Op18b (14.6-fold). The expression of unphosphorylated Op18 was found to be strongly correlated with both phosphorylated isoforms Op18a and Op18b (r = 0.49 and 0.56, respectively; p < 0.05) by Spearman correlation coefficient analysis (Fig. 1, B and C). The phosphorylated isoform Op18a was also strongly correlated with the phosphorylated isoform Op18b (r = 0.42, p < 0.05).

Overexpression of Op18 and Op18b in Poorly Differentiated Lung Adenocarcinomas—Op18a and Op18b could be detected in over 70% of the poor or moderately differentiated lung adenocarcinomas, whereas Op18b could be detected in only 31.8%, and Op18a could be detected in only 50% in the 22 well differentiated tumors. Op18b was detected in 12.8–36.4% of these three differentiation groups. Quantitative analysis revealed Op18b and Op18b to be significantly increased in poorly differentiated adenocarcinomas as compared with either moderate or well differentiated tumors (p < 0.03). Op18a did not show a significant relationship with tumor differentiation (Table II). The unphosphorylated Op18 was also found to be overexpressed in bronchial derived adenocarcinomas as compared with bronchioloalveolar tumors (p = 0.02). No significant differences were found between Op18 expression and...
Increased Transcription Underlies Elevated Op18 Protein Expression

— To examine whether the changes in protein expression were due to transcriptional or other mechanisms of regulation, the expression of Op18 mRNA within the same lung samples (76 tumors and nine normal lung tissues for which both mRNA and protein data were available) was examined using oligonucleotide microarrays. A strong correlation was observed between the mRNA expression levels and the different protein isoforms Op18, Op18a, and Op18b ($r = 0.533, 0.398,$ and $0.383,$ respectively). A strong correlation was also observed for Op18 mRNA expression among tumors of different stage and tumor differentiation indicating the expression of Op18 mRNA is highly correlated to the level of these protein isoforms (data not shown). Op18 mRNA was also significantly increased in lung adenocarcinomas relative to uninvolved normal lung, although mRNA levels showed only a 1.4-fold relative increase in the tumors (Table I). This suggests that the increase in Op18 protein expression in the lung tumors is associated with a corresponding increase in its mRNA, and thus Op18 protein expression is likely to be transcriptionally regulated.

**Relationship between Op18 and clinicopathologic variables in lung adenocarcinomas**

| Variables                               | n  | Op18 | Op18a | Op18b |
|-----------------------------------------|----|------|-------|-------|
| Age                                     |    |      |       |       |
| <65                                     | 49 | 0.847 | 0.4124| 0.7775|
| >65                                     | 44 | 0.606 | 0.039 up$^a$| 0.3148|
| Gender                                  |    |      |       |       |
| Female                                  | 53 | 0.778 | 0.7075| 0.2494|
| Male                                    | 40 |       |       |       |
| Smoking                                 |    |      |       |       |
| Smoking                                 | 79 | 0.66  | 0.9621| 0.5595|
| Nonsmoking                              | 10 |       |       |       |
| Stage                                   |    |      |       |       |
| Stage I                                 | 64 | 0.04 down$^a$| 0.1594| 0.5576|
| Stage III                               | 29 |      |       |       |
| T status                                |    |      |       |       |
| T1                                      | 49 | 0.616 | 0.6959| 0.7476|
| T2–T4                                   | 44 | 0.026 down | 0.3043 | 0.6912 |
| N status                                |    |      |       |       |
| N0                                      | 68 | 0.0001 up | 0.1897 | 0.0364 up |
| N1, N2                                  | 25 |      |       |       |
| Classification                          |    |      |       |       |
| Bronchioloalveolar                      | 14 | 0.495 | 0.5068| 0.9308|
| Bronchial derived                       | 76 | 0.051 | 0.6924| 0.7396|
| Differentiation                         |    |      |       |       |
| Poor                                    | 23 | 0.286 | 0.6886| 0.3825|
| Moderate                                | 47 |      |       |       |
| Well                                    | 22 |      |       |       |
| Lymphocytic response                    |    |      |       |       |
| Yes                                     | 41 | 0.25  | 0.25  | 0.25  |
| No                                      | 52 | 0.051 | 0.6924| 0.7396|
| p53 nuclear accumulation                |    |      |       |       |
| Positive                                | 28 | 0.286 | 0.6886| 0.3825|
| Negative                                | 54 |      |       |       |
| K-ras 12th/13th codon mutation          |    |      |       |       |
| Positive                                | 36 | 0.286 | 0.6886| 0.3825|
| Negative                                | 40 |      |       |       |

* $p$ value of $F$ test: up, increased in female; down, decreased in T1.

Patient survival, tumor stage, patient smoking history, lymphocytic response, p53 nuclear accumulation, or K-ras 12th/13th codon mutational status.

**Increased Transcription Underlies Elevated Op18 Protein Expression**—To examine whether the changes in protein expression were due to transcriptional or other mechanisms of regulation, the expression of Op18 mRNA within the same lung samples (76 tumors and nine normal lung tissues for which both mRNA and protein data were available) was examined using oligonucleotide microarrays. A strong correlation was observed between the mRNA expression levels and the different protein isoforms Op18, Op18a, and Op18b ($r = 0.533, 0.398,$ and $0.383,$ respectively). A strong correlation was also observed for Op18 mRNA expression among tumors of different stage and tumor differentiation indicating the expression of Op18 mRNA is highly correlated to the level of these protein isoforms (data not shown). Op18 mRNA was also significantly increased in lung adenocarcinomas relative to uninvolved normal lung, although mRNA levels showed only a 1.4-fold relative increase in the tumors (Table I). This suggests that the increase in Op18 protein expression in the lung tumors is associated with a corresponding increase in its mRNA, and thus Op18 protein expression is likely to be transcriptionally regulated.

**Relationship between Op18/Op18b and Other Proteins Identified in Lung Adenocarcinoma**—Table III lists proteins positively correlated with Op18/Op18b protein expression in this tumor series as determined using Spearman correlation analysis. These proteins appear to be involved in a number of diverse pathways including carbohydrate or protein metabolism, cell structure, and signal transduction. Most of these proteins were also increased in poorly differentiated tumors. Glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate 1-dehydrogenase variant A, and cytokeratin 8 were correlated with the unphosphorylated Op18; however, cytokeratin 18, 26 S proteasome p28, and the proteasome activator PA28 subunit $\beta$ were significantly correlated with both the unphosphorylated Op18 and phosphorylated Op18b.

**Table III**

| Relationship between Op18 and clinicopathologic variables in lung adenocarcinomas | n  | Op18 | Op18a | Op18b |
|----------------------------------------------------------------------------------|----|------|-------|-------|
| Age                                                                              |    |      |       |       |
| <65                                                                              | 49 | 0.847 | 0.4124| 0.7775|
| >65                                                                              | 44 | 0.606 | 0.039 up$^a$| 0.3148|
| Gender                                                                           |    |      |       |       |
| Female                                                                           | 53 | 0.778 | 0.7075| 0.2494|
| Male                                                                             | 40 |      |       |       |
| Smoking                                                                          |    |      |       |       |
| Smoking                                                                          | 79 | 0.66  | 0.9621| 0.5595|
| Nonsmoking                                                                       | 10 |      |       |       |
| Stage                                                                            |    |      |       |       |
| Stage I                                                                          | 64 | 0.04 down$^a$| 0.1594| 0.5576|
| Stage III                                                                       | 29 |      |       |       |
| T status                                                                         |    |      |       |       |
| T1                                                                               | 49 | 0.616 | 0.6959| 0.7476|
| T2–T4                                                                           | 44 | 0.026 down | 0.3043 | 0.6912 |
| N status                                                                         |    |      |       |       |
| N0                                                                               | 68 | 0.0001 up | 0.1897 | 0.0364 up |
| N1, N2                                                                           | 25 |      |       |       |
| Classification                                                                   |    |      |       |       |
| Bronchioloalveolar                                                              | 14 | 0.495 | 0.5068| 0.9308|
| Bronchial derived                                                               | 76 | 0.051 | 0.6924| 0.7396|
| Differentiation                                                                 |    |      |       |       |
| Poor                                                                             | 23 | 0.286 | 0.6886| 0.3825|
| Moderate                                                                        | 47 |      |       |       |
| Well                                                                             | 22 |      |       |       |
| Lymphocytic response                                                            |    |      |       |       |
| Yes                                                                              | 41 | 0.25  | 0.25  | 0.25  |
| No                                                                               | 52 | 0.051 | 0.6924| 0.7396|
| p53 nuclear accumulation                                                        |    |      |       |       |
| Positive                                                                        | 28 | 0.286 | 0.6886| 0.3825|
| Negative                                                                        | 54 |      |       |       |
| K-ras 12th/13th codon mutation                                                   |    |      |       |       |
| Positive                                                                        | 36 | 0.286 | 0.6886| 0.3825|
| Negative                                                                        | 40 |      |       |       |

* $p$ value of $F$ test: up, increased in female; down, decreased in T1.
Op18 Expression in Lung Adenocarcinomas

**Table III**

| Spot no. | Op18b | Op18 |
|----------|-------|------|
| 947      | 0.18a | 0.27 |
| 450      | 0.19  | 0.27 |
| 352      | 0.18  | 0.33 |
| 514      | 0.30  | 0.30 |
| 1252     | 0.28  | 0.27 |
| 1062     | 0.29  | 0.27 |
| 702      | 0.37  | 0.12 |
| 152      | 0.30  | 0.13 |
| 935      | 0.28  | 0.15 |

**Correlation between Op18/Op18b and other proteins expressed in lung adenocarcinomas**

**Positive correlation**

- Glyceraldehyde-3-phosphate dehydrogenase: Carbohydrate metabolism (0.27)
- Glucose-6-phosphate 1-dehydrogenase variant A: Carbohydrate metabolism (0.33)
- Cytokeratin 18: Cell structure (0.30)
- Cytokeratin 8: Cell structure (0.33)
- Annexin IV: Phospholipase inhibitor (0.26)
- Annexin V: Phospholipase inhibitor (0.27)
- Protein disulfide isomerase precursor: Protein folding (0.12)
- Protein disulfide isomerase precursor: Protein folding (0.27)
- Transthyretin: Small molecule transport (0.36)
- Ferritin light chain: Small molecule-binding protein (0.39)

**Negative correlation**

- Annexin IV: Phospholipase inhibitor (-0.08)
- Annexin V: Phospholipase inhibitor (-0.07)
- Fibroblast growth factor-4: Cell proliferation (-0.31)
- Transthyretin: Small molecule transport (-0.36)
- Ferritin light chain: Small molecule-binding protein (-0.39)

*S* Spearman correlation coefficient based on 93 lung tumor samples. Bold values indicate significant correlation, *p* < 0.05.

Table: Oxidoreductases in Lung Adenocarcinomas

- SOD2: Superoxide dismutase (0.28)
- GRP78: Glucose-6-phosphate 1-dehydrogenase variant A (0.27)
- 14-3-3: Small molecule-binding protein (0.18)

**DISCUSSION**

Specific markers for lung cancer have been the subject of extensive research. A number of markers have been examined including neuron-specific enolase, carcinoembryonic antigen, cytokeratin 19 fragments, cancer antigen CA 125, and tissue polypeptide antigen (33). In contrast to the utilization of a single marker, multiple biological markers may ultimately prove more informative due to the known heterogeneity of lung cancers (34, 35).

Elevated expression of Op18 was initially reported in malignant blood cells (36) and subsequently observed in other tumor types including ovarian and breast cancers (16–18). Op18 expression appears to be linked to cell proliferation; however, this may depend on the particular cell/tissue/tumor system studied (17). Consistent with this proliferation link,
Nishio et al. (37) have reported that Op18 expression was associated with lung carcinoma cell sensitivity to vindesine and vincristine. In the present study, we analyzed the expression of the different isoforms of Op18 protein as well as its mRNA expression in a large series of human lung adenocarcinomas using quantitative 2D PAGE analysis, 2D Western blot, immunohistochemistry, and oligonucleotide microarrays. Three phosphorylated and one unphosphorylated forms of Op18 were identified on 2D gels in lung adenocarcinomas. The two major phosphorylated isoforms Op18a and Op18b have been previously reported as being phosphorylated at Ser-25 for Op18a and Ser-25 and Ser-38 for Op18b (26–29). The pattern of Op18 expression among these isoforms in lung adenocarcinomas is different from that observed in acute leukemia, which expresses three isoforms (12), and a pre-B 697 line, which expresses seven isoforms (38). In the lung tumors examined in the present study, the abundance of the unphosphorylated Op18 isoform was much higher than the abundance of the other three phos-

| Table IV |

Correlation between Op18 and other gene mRNAs expressed in lung adenocarcinomas

| Gene name | Functions |
|-----------|-----------|
| TUBB      | Tubulin-cytoskeleton-associated |
| CDC20     | Cell cycle control, mitotic spindle checkpoint |
| TYMS      | Nucleotide metabolism |
| SNRPD2    | RNA processing, mRNA splicing |
| CDC25C    | Cell cycle control, protein tyrosine phosphatase |
| CHAF1A    | DNA replication-dependent nucleosome assembly |
| HDAC2     | Transcription factor |
| NASP      | DNA packaging |
| LMB1      | Mitotic S-specific transcription, structural protein |
| TOP2A     | DNA synthesis |
| MCM4      | DNA synthesis |
| KNSL6     | Cell proliferation, mitosis |
| PCNA      | Cell proliferation, cell cycle control, DNA replication |
| MYBL2     | Transcription factor, cell cycle control |
| MCM2      | DNA synthesis |
| NEK2      | Protein kinase, cell cycle control |
| CENPF     | Mitosis |
| MCM3      | DNA synthesis |
| FOXM1     | Cell proliferation, transcription factor |
| UBC110    | Cell proliferation |
| TROAP     | Cell adhesion |
| TTK       | Cell proliferation, protein tyrosine kinase |
| CDKN2A    | Cell cycle control, cyclin-dependent protein kinase inhibitor |
| PSMB5     | Protein degradation |
| FYN       | Protein tyrosine phosphatase |
| CDKN3     | Cell cycle control, protein phosphatase |
| KNSL1     | Mitosis, mitotic spindle assembly |
| LMB12     | Mitotic S-specific transcription, structural protein |
| CCNA2     | Cell cycle control, regulation of CDK activity |
| E2F5      | Cell proliferation, transcription factor |
| CENPA     | DNA-binding protein |
| PSMB2     | Protein degradation |
| CSE1L     | Cell proliferation, apoptosis |
| RANBP1    | Signal transduction |
| CDP2      | Protein kinase, cell proliferation, cell cycle control |
| KAP1      | Tubulin-cytoskeleton-associated |
| PSMD2     | Protein degradation |
| CCNF      | Cell cycle control, regulation of CDK activity |
| MEST      | Central nervous system development |

*N* p value between poor (P) versus well (W) differentiated tumor. Bold values indicate significant correlation, *p < 0.05*. False discovery rate = 9.5%.
phorylated forms, yet a strong correlation was observed between the expression of all forms, indicating that the subsequent modification of the phosphorylated isoforms is directly related to the overall level of the unphosphorylated form.

Quantitative analysis indicated that all three Op18 isoforms (Op18, Op18a, and Op18b) were significantly increased in lung tumors relative to normal lung. Immunohistochemical analysis of tissue microarrays confirmed abundant cytoplasmic Op18 protein staining in lung tumors relative to normal lung, and a good correlation was observed between the expression level indicated by tissue microarrays and the level determined by quantitative 2D PAGE analysis. The increased abundance of the phosphorylated isoforms in the lung tumors (from 3.2% in normal to 7.9% in tumor) potentially indicates a higher level of progression through the cell cycle in the tumors.
as higher order phosphorylation is associated with G2/M (39). The function of Op18 has been previously speculated to be involved in cell proliferation and differentiation (36). A high expression level of Op18 is observed in poorly differentiated prostate adenocarcinoma and ovarian and breast cancers (15–17). We observed that both the phosphorylated and unphosphorylated Op18 isoforms were significantly increased in poorly differentiated lung adenocarcinomas as compared with the moderate or well differentiated tumors, supporting earlier observations in other tumor types. Moreover we observed that the Op18 mRNA expression was correlated to cell proliferation-related genes such as PCNA, CSE1L, FOXM1, TTK, and UBCH10 (40–42), which were also significantly increased in poorly differentiated adenocarcinomas. The unphosphorylated form of Op18 was also found to be overexpressed in bronchial derived adenocarcinomas, which may be indicative of higher proliferation rates in the former tumors. No other significant relationships between Op18 and patient survival, tumor stage, smoking status, lymphocytic response, or K-ras 12th/13th codon mutational status were observed in this tumor cohort.

Many genes have been reported to affect the expression and phosphorylation of the Op18 protein. For example, transcription factors (E2F, AP-2, and Sp1) can increase Op18 expression (38), whereas p53 appears to repress the Op18 expression (43). Protein kinases such as cyclin-dependent kinases (CDKs), the Ca2+/calmodulin-dependent kinase IV/Gr can cause extensive phosphorylation of Op18 protein indicating that Op18 can be influenced by a number of signal transduction pathways (10). We found that the mRNA expression of the transcription factor E2F5 and the protein kinase CDK2 were strongly correlated with Op18 mRNA levels in poorly differentiated bronchial derived adenocarcinomas. Tubulin is also considered a potential downstream target of the Op18 signal pathways (44). We found that the tubulin-associated gene TUBB was strongly correlated with Op18 mRNA expression in adenocarcinomas of different cell derivation and differentiation. CKAP1, another tubulin-associated gene, was strongly correlated with Op18 levels only in poorly differentiated bronchial derived tumors. We did not find Op18 to be correlated with p53 at either the mRNA or protein level in lung adenocarcinomas (data not shown). Some genes were not significantly correlated with p53 at either the mRNA or protein level in lung adenocarcinomas (data not shown). Some genes were not significantly associated with Op18 expression in the well differentiated or bronchioloalveolar tumors, such as NSEP1, MEST, CCNF, PSMD2, RANBP1, CSE1L, PSM2B, CENPA, CCNA2, RFC2, LMNB2, and KNSL1, although these genes maintained a strong correlation with Op18 in poorly differentiated tumors. This may indicate different regulatory mechanisms for Op18-related processes in tumors of different derivation or cell differentiation.

Cell differentiation in lung cells can be induced by treatment with DX, IL-6, or IFN-α (31, 32). Higher levels of Op18 were found in the poorly differentiated SKLU1 cells as compared with the well differentiated A549 cells. Op18 levels were decreased in well differentiated cells after IL-6 treatment, but Op18a was increased after IL-6 treatment indicating that Op18 phosphorylation may be associated with events leading to cell differentiation. Cell proliferation induced following serum increased the levels of both Op18 and Op18b in the well differentiated A549 cells but not in the poorly differentiated SKLU1 cells. The expression of Ki-67 is correlated with cell proliferative status (45). We found that Op18 and Op18b levels were significantly correlated with Ki-67 levels (p < 0.05) in the same lung tumor tissues using immunohistochemistry (data not shown). These results suggest that both Op18 and phosphorylated Op18 are involved in cell proliferation, and the link between Op18 expression and cell proliferation may depend on the differentiation status of the cell or tissue.

The analyses performed in this study suggest that the expression of Op18 protein in lung cancer is regulated at the transcriptional level. Overexpression of Op18 proteins in lung adenocarcinomas, especially in poorly differentiated tumors, indicates that Op18 may be useful as a potential tumor marker. The role of different isoforms of Op18 in microtubule regulation and tumor development and its association with other genes in lung cancer identified in this study will require further investigation. The increased expression of the phosphorylated forms of Op18 in these lung tumors may offer a new target for drug or gene therapy, which may help to increase patient survival.

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