A Novel Human tRNA-Dihydrouridine Synthase Involved in Pulmonary Carcinogenesis

Tatsuya Kato,1,2 Yataro Daigo,1 Satoshi Hayama,1 Nobuhisa Ishikawa,1 Takumi Yamabuki,1 Tomoo Ito,1 Masaki Miyamoto,1 Satoshi Kondo,1 and Yusuke Nakamura1

Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan and Departments of Surgical Oncology and Surgical Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Abstract

An increased level of dihydrouridine in tRNA\textsuperscript{Phe} was found in human malignant tissues nearly three decades ago, but its biological significance in carcinogenesis has remained unclear. Through analysis of genome-wide gene-expression profiles among non–small cell lung carcinomas (NSCLC), we identified overexpression of a novel human gene, termed hDUS2, encoding a protein that shared structural features with tRNA-dihydrouridine synthases (DUS). The deduced 493-amino-acid sequence showed 39% homology to the dihydrouridine synthase 2 enzyme (Dus2) of \textit{Saccharomyces cerevisiae} and contained a conserved double-strand RNA-binding motif (DSRM). We found that hDUS2 protein had tRNA-DUS activity and that it physically interacted with EPRS, a glutamyl-prolyl tRNA synthetase, and was likely to enhance translational efficiencies. A small interfering RNA against hDUS2 transfected into NSCLC cells suppressed expression of the gene, reduced the amount of dihydrouridine in tRNA molecules, and suppressed growth. Immunohistochemical analysis showed significant association between higher levels of hDUS2 in tumors and poorer prognosis of lung cancer patients. Our data imply that up-regulation of hDUS2 is a relatively common feature of pulmonary carcinogenesis and that selective suppression of hDUS2 enzyme activity and/or inhibition of formation of the hDUS2-tRNA synthetase complex could be a promising therapeutic strategy for treatment of many lung cancers.

(Cancer Res 2005; 65(13): 5638-46)

Introduction

Lung cancer is one of the most common causes of cancer death worldwide, and non–small cell lung cancer (NSCLC) accounts for nearly 80% of those cases (1). Many genetic alterations associated with development and progression of lung cancer have been reported, but the precise molecular mechanisms remain unclear (2). Over the last decade, newly developed cytotoxic agents, including paclitaxel, docetaxel, gemcitabine, and vinorelbine, have emerged to offer multiple therapeutic choices for patients with lung cancer; however, those regimens provide only modest benefit and response rates (2). Over the last decade, newly developed cytotoxic agents, including paclitaxel, docetaxel, gemcitabine, and vinorelbine, have emerged to offer multiple therapeutic choices for patients with lung cancer.

Materials and Methods

Lung cancer cell lines and tissue samples. The human lung cancer cell lines used in this study were as follows: lung adenocarcinomas A427, A549, LC319, PC3, PC9, PC14, NCI-H23, NCI-H522, and NCI-H1355; bronchioalveolar cell carcinomas NCI-H1666 and NCI-H1781; lung adenosquamous carcinomas NCI-H226 and NCI-H647; lung squamous cell carcinomas (SCC) RERF-LC-AI, SK-MES-1, EBC-1, LC176, LU61, NCI-H520, NCI-H1703, and NCI-H1710; a lung large cell carcinoma (LCC) LX1; and small cell lung cancers (SCLC) DMS114, DMS273, SBC-3, and SBC-5. All cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% \textit{CO}_2. Human small airway epithelial cells (SAEC) were grown in optimized medium (SAGM) purchased from Cambrex Bio Science, Inc. (Walkersville, MD). Primary NSCLC samples, of which 22 were classified as adenocarcinomas, 11 as SCCs, and one as adenosquamous carcinoma, were obtained from 37 patients with written informed consent, as described previously (5).

Requests for reprints: Yusuke Nakamura, Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-Ward, Tokyo 108-8639, Japan. Phone: 81-3-5449-5372; Fax: 81-3-5449-5433; E-mail: yusuke@ims.u-tokyo.ac.jp. ©2005 American Association for Cancer Research.
tissue microarray and additional statistical analysis were also obtained from patients who underwent surgery at Hokkaido University and its affiliated hospitals.

Semiquantitative reverse transcription-PCR. Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol. Extracted RNAs and normal human tissue polyadenylate acid [poly(A)] RNAs were treated with DNase I (Nippon Gene, Tokyo, Japan) and reversely transcribed using oligo (dT) primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following synthesized hDUS2-specific primers or with ACTB-specific primers as an internal control: hDUS2, 5’-GACCACATCAACAGTTTGC-3’ and 5’-TGCCACGACATCTAATCTCTG-3’; ACTB, 5’-GAGGTTGATAGCATTGTCTTCG-3’ and 5’-CAAGTCAGTGTACAGTGTAAC-3’. PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Northern blot analysis. Human multiple-tissue blots (BD Biosciences Clontech, Palo Alto, CA) and lung cancer cell blots containing mRNA from four NSCLC cell lines (NCI-H23, NCI-H522, LC176, and LC319) and four normal organs were hybridized with a 32P-labeled PCR product of hDUS2. The cdNA probes of hDUS2 were prepared by RT-PCR using the primers described above. Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed at room temperature for 30 hours with intensifying BAS screens (Bio-Rad, Hercules, CA).

Isolation and DNA sequencing of cdNA. Among genes that were overexpressed in the majority of the NSCLCs examined on our cdNA microarray, we identified one clone, later termed hDUS2, that seemed of special interest. To obtain a full-length cdNA of the transcript, we screened the BLAST database using this fragment as a query sequence and identified several expressed sequence tags (EST) containing the 5’ portion of the unknown gene. After assembling the DNA sequences of these clones, the entire coding region of human hDUS2 was amplified by RT-PCR, using forward primer of either 5’-GCTGTAACACAGGAGGAATG-3’ (for pcDNA-hDUS2 and pcAGGS-n3FH-hDUS2) or 5’-ATGATGTGTTAATGCACTCCT-3’ (for pcAGGS-n3FH-hDUS2) and reverse primer of 5’-CCACGCTTCCAGGG-GGT-3’ (for all the three vectors), and subsequently cloned into appropriate sites of individual expression plasmids: pcDNA3.1-MyHC (Invitrogen; pcDNA-hDUS2), COOH-terminal FLAG-tagged-pcAGGS (pcAGGS-n3FH-hDUS2), and NH2-terminal FLAG-tagged and COOH-termini HA-tagged-pcAGGS vectors (pcDNA3.1-hDUS2). The cdNA sequence of each cdNA clone was determined with an ABI Prism 3700 DNA Sequencer (Applied Biosystems, Foster City, CA), using T3, T7, or synthetic oligonucleotide primers according to the manufacturer’s instructions.

Anti-hDUS2 antibodies. Plasmids expressing partial fragments of hDUS2 (cdons 15-338) that contained His-tagged epitopes at their NH2-terminals were prepared using pET28 vector (Novagen, Madison, WI). The recombinant peptides were expressed in Escherichia coli, BL21 codon-plus strain (Stratagene, La Jolla, CA), and purified using TALON resin (BD Biosciences Clontech) according to the supplier’s protocol. The protein, extracted on an SDS-PAGE gel, was inoculated into rabbits; the immune sera were purified on affinity columns according to standard methodology. Affinity-purified anti-hDUS2 antibodies were used for Western blotting, immunoprecipitation, and immunostaining. On Western blots, we confirmed that the antibody was specific to hDUS2, using lysates from NSCLC cell lines that either expressed hDUS2 endogenously or not, or cells that had been transfected with hDUS2 expression vector.

RNA interference assay. We had previously established a vector-based RNA interference (RNAi) system, psiH1X30, that was designed to synthesize small interfering RNAs (siRNA) in mammalian cells (6); 10 μg of siRNA-expression vector was transfected using 30 μL of LipofectAMINE 2000 (Invitrogen) into NSCLC cell lines, A549 and LC319. The transfected cells were cultured for 7 days in the presence of appropriate concentrations of geneticin (G418), and the number of colonies was counted by Giemsa staining, and viability of cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 7 days after the treatment. Briefly, cell-counting kit-8 solution (DOJINDO, Kumamoto, Japan) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for additional 4 hours. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 550 (Bio-Rad). To confirm suppression of hDUS2 protein expression, Western blotting was carried out with affinity-purified hDUS2 polyclonal antibody according to the standard protocol. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control 1 (mock vector); control 2 (Scramble: chloroplast Euglena gracilis gene coding for 5S and 16S rRNAs), 5’-GCCCCGTTTGTAGATTTTCG-3’; control 3 (Luciferase, Photinus pyralis luciferase gene), 5’-CTACGCGGGAATACCTTGCA-3’; siRNA-hDUS2-#1, 5’-GATCTCAGACTCCTTGTT-3’; siRNA-hDUS2-#2, 5’-GTTGGGCA-CAGCTTGAT-3’.

Identification of hDUS2-associated proteins. Cell extracts from lung cancer cell line LC319, transfected with plasmids expressing hDUS2 (pcAGGS-n3FH-hDUS2) or mock vector (control), were precleared by incubation at 4°C for 1 hour with 100 μL of protein G-agarose beads, in final volumes of 2 mL of immunoprecipitation buffer (0.5% NP40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of protease inhibitor. After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with anti-FLAG M2 agarose for 2 hours. After the beads were collected from each sample by centrifugation at 5,000 rpm for 2 minutes and washed six times with 1 mL of protein G-agarose buffer, they were eluted by FLAG-peptide (Sigma-Aldrich Co., St. Louis, MO). Eluted material was incubated at 4°C with anti-HA agarose for 2 hours, and the washed beads were resuspended in 50 μL of Laemmli sample buffer and boiled for 5 minutes before the proteins were separated on 5% to 10% SDS-PAGE gels (Bio-Rad). After electrophoresis, the gels were stained with silver. Protein bands found specifically in hDUS2-transfected extracts were excised to serve for analysis by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS; AXIMA-CFR plus, Shimadzu Bio-TECH, Kyoto, Japan). To confirm the interaction between hDUS2 and EPRS (a glutamyl-prolyl tRNA synthetase), we carried out the immunoprecipitation experiment. To achieve FLAG-tagged EPRS, we cloned the entire coding sequence into the appropriate site of pXFXL-FLAG-CMV10 plasmid vector (Sigma-Aldrich, pcMV10-EPRS). The extracts from LC319 cell transfected with pcMV10-EPRS were immunoprecipitated with affinity-purified hDUS2 polyclonal antibody and normal rabbit IgG, respectively. Immunoblot was developed using anti-FLAG M2 antibody (Sigma-Aldrich, pcMV10-EPRS) with a horseradish peroxidase-labeled secondary antibody (Sigma-Aldrich, pcMV10-EPRS) according to the standard protocol.

Isolation of tRNA from human cells. Total RNAs from A549 (human lung adenocarcinoma) cells were isolated by phenol extraction and DEAE column chromatography. A549 cells transfected with plasmids expressing hDUS2 (pcAGGS-n3FH-hDUS2) and mock vector were cultured for 2 days without G418 selection. The level of transfaction into A549 cells detected by flow cytometric analysis was 70% when we transfected pEGFP vector into the cells using LipofectAMINE 2000 (Invitrogen) reagent. After samples were collected by centrifugation at 1,200 rpm for 5 minutes and washed twice with PBS, the cells were suspended in 2 mL of buffer A [20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 1 mol/L NaCl, 0.5 mmol/L EDTA] and shaken for 15 minutes in a centrifuge tube with an equal volume of 88% phenol saturated with buffer A. The aqueous layer was mixed with 2.5 volumes of ethanol. Precipitates were collected by centrifugation and dissolved in RNase-free water. After DNase I treatment (Nippon Gene), each solution was applied to a column of TSK gel DEAE-SPW (TOSOH, Tokyo, Japan) and eluted with buffer B [0.3 mol/L NaCl, 100 mmol/L Tris-HCl (pH 7.5) in flow-through fractions. Before use, total tRNAs were precipitated by addition of 2.5 volumes of ethanol at ~80°C.

Assay for tRNA dihydrouridinylase. Colorimetric measurement of tRNA dihydrouridinylase was done according to the method of Jacobson and Hedgcock (13). Briefly, a solution containing 25 μg of total tRNA, measured in microcells with a spectrophotometer (DU-640, Beckman Coulter, Inc., Fullerton, CA), was treated with alkali (KOH) to convert dihydrouridine to its open-ring form (N-ribosyl-3-ureidopropionic acid) and quantified by a modified colorimetric assay specific for ureido groups (14). We placed each
sample of total tRNA to be assayed in a tube and added RNase-free water to make up 100 μL. Then 10 μL of 1 N KOH were added to each reaction mixture and the mixtures were maintained for 30 minutes at 37°C. Next, we added 50 μL of concentrated H₂SO₄ and 100 μL of a mixture of N-phenyl-p-phenylenediamine (Sigma-Aldrich) and 2,3-butadione monoxime (Sigma-Aldrich), prepared as described by Hunninghake and Grisolia (14) except that the free base (N-phenyl-p-phenylenediamine) was used instead of the hydrochloride salt. After heating for 5 minutes at 95°C, this mixture was transferred to a 50°C water bath and incubated for at least 5 minutes before addition of 100 μL of a solution of 0.001 mol/L FeCl₃ in concentrated H₂SO₄. After cooling, the A₅₅₀ nm of each solution was determined in microcells with a spectrophotometer.
Effect of hDUS2 on in vitro translation efficiency. A rabbit-
reticulocyte lysate system was purchased from Promega Corp. (Madison,
WI). Aliquots of each reaction tube containing hDUS2 recombinant protein
and/or bovine serum albumin (BSA) were mixed with 35 μL of rabbit-
reticulocyte lysate, 1 μL of an amino acid mixture minus methionine, 2 μL
of 35S methionine (1,200 Ci/mmol), 2 μL of RNase inhibitor (40 units/μL,
TAKARA, Tokyo, Japan), and 0.25 μg of poly(A) RNA from A549 cells that
had been denatured at 65°C for 3 minutes. The reactions were cooled
immediately on ice. Recombinant glutathione S-transferase (GST)–fused
hDUS2 protein produced by E. coli was purified using Glutathione
Sepharose 4B according to the manufacturer’s protocols. The GST-hDUS2
protein (3.6 pmol), BSA (3.6 pmol, from BSA solution in DNase kit (RNase-
free and Reverse Transcriptase option Grade; Nippon Gene)), and 0.25 μL
mixtures of both proteins in final doses of 3.6 pmol proteins (hDUS2 versus
BSA, 100/9, 50/50, 0:100; individually) were added to each reaction mixture.
Translation reactions were started immediately by incubation at 30°C for
90 minutes. The results of translation were analyzed by SDS-PAGE according
to the standard protocol. BSA used in this assay were confirmed
in advance to have no inhibitory effect on in vitro translation or RNA
stability by addition of BSA to the reaction mixture, which never decreased
the final amount of translated protein detected on SDS-PAGE analysis,
when compared with the protein level after addition of only PBS to the
mixture (data not shown).

Immunohistochemistry and tissue microarray analysis. Tumor tissue
microarrays were constructed as published elsewhere, using formalin-fixed
NSCLCs (15). Tissue areas for sampling were selected based on visual
alignment with the corresponding H&E-stained sections on slides. Three,
four, or five tissue cores (diameter, 0.6 mm; height, 3-4 mm) taken from
donor tumor blocks were placed into recipient paraffin blocks using a tissue
microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue
was punched from each case. Five-micrometer sections of the resulting
microarray block were used for immunohistochemical analysis. Positivity for
hDUS2 was assessed semiquantitatively by three independent investigators
without prior knowledge of the clinical follow-up data, each of whom
recorded staining intensity as absent (scored as 0), weak (1+), or strongly
positive (2+). Lung cancers were scored as positive (2+) only if all reviewers
defined them as such. To investigate the presence of hDUS2 protein in
clinical materials, we stained tissue sections using ENVISION+ Kit/HRP
(DakoCytomation, Glostrup, Denmark). Affinity-purified anti-hDUS2 anti-
body was added after blocking of endogenous peroxidase and proteins, and
each section was incubated with horseradish peroxidase–labeled anti-rabbit
immunoglobulin G as the secondary antibody. Substrate chromogen was
added and the specimens were counterstained with hematoxylin.

Statistical analysis. We attempted to correlate clinicopathologic
variables such as age, gender, and pathologic tumor-node-metastasis stage
with the expression levels of hDUS2 protein determined by tissue microarray
analysis. Tumor-specific survival curves were calculated from the date of
surgery to the time of death related to NSCLC, or to the last follow-up
observation. Kaplan-Meier curves were calculated for each relevant variable
and for hDUS2 expression; differences in survival times among patient
subgroups were analyzed using the log-rank test. Univariate and multivariate
analyses were done with the Cox proportional-hazard regression model to
determine associations between clinicopathologic variables and cancer-
related mortality. First, we analyzed associations between death and possible
prognostic factors including age, gender, pT classification, and pN
classification, taking into consideration one factor at a time. Second,
multivariate Cox analysis was applied on backward (stepwise) procedures
that always forced hDUS2 expression into the model, along with any and all
variables that satisfied an entry level of a P < 0.05. As the model continued to
add factors, independent factors did not exceed an exit level of P < 0.05.

Results
Identification of the hDUS2 gene. Using a cDNA microarray
representing 23,040 genes to screen for elements that are highly
transactivated in a large proportion of NSCLCs, we identified cDNA
fragment, later termed hDUS2, which corresponded to one
unannotated transcript (FLJ20399, accession no. NM_017803).
This gene showed a 3-fold or higher level of expression in the
great majority of the NSCLC cases we examined. Subsequently
we confirmed its transactivation by semiquantitative RT-PCR

Figure 2. Structural features of hDUS2. A, alignment of the predicted amino
acid sequences of human hDUS2 and DUS from various other species. Shading
indicates homologous residues. B, schematic structure of hDUS2 protein.
A Dus domain at the NH2-terminal end and the DSRM at the COOH-terminal are
both conserved in the protein structure.
experiments in 11 of 12 additional NSCLC cases (five of six adenocarcinomas; all of six SCCs; Fig. 1A). Up-regulation of hDUS2 was observed in 21 of the 23 NSCLC and SCLC cell lines examined, whereas the transcript was hardly detectable in SAEC cells derived from normal bronchial epithelium (Fig. 1B, left). We confirmed expression of hDUS2 in lung tumors by examining endogenous expression of hDUS2 protein on Western blots using anti-hDUS2 antibody in 5 NSCLC and SAEC cells (Fig. 1B, right). Northern blot analysis using hDUS2 cDNA as a probe identified a 2.3-kb transcript; weak signals could clearly only be identified in heart, placenta, and skeletal muscle, among the 23 normal human tissues examined (Fig. 1C, left). Although a 3.0-kb transcript was observed exclusively and abundantly in testis, that transcript was not expressed in any other tissues examined. Additional Northern blotting using the same probe detected only the 2.3-kb transcript in lung cancer cells, much more abundantly than normal lung tissue (Fig. 1C, right). Hence, we focused on this 2.3-kb transcript for further analysis. To obtain a full-length cDNA sequence, we screened the BLAST database and identified several ESTs containing the 5' part of this gene. Assembly of DNA sequences for these clones and subsequent cloning of the
RT-PCR products determined the entire coding sequence; the cDNA consisted of 2,008 nucleotides, with an open reading frame of 1,479 nucleotides encoding a 493-amino-acid peptide. A homology search using the BLAST program indicated homology with proteins belonging to the family of tRNA-dihydrouridine synthases (DUS); it showed 39% identity in amino acid sequence to Dus2 of *S. cerevisiae*, which catalyzes reduction of the 5,6-double bond of a uridine residue in tRNA (Fig. 2A). Hence, we designated this gene hDUS2 (accession no. AB101210). Analysis with the SMART program identified two motifs: a Dus domain in the NH2-terminal region and a double-strand RNA-binding motif (DSRM) domain in the COOH-terminal (Fig. 2B). Human DUS2 seemed to have 90% identity in amino acids with its murine homologues (*Mus musculus* and *Rattus norvegicus*) and 48% and 40% respectively with DUS enzymes in *Drosophila melanogaster* and *Caenorhabditis elegans* (Fig. 2A). To determine the subcellular localization of endogenous hDUS2 in lung cancer cells, we used immunocytochemical analysis using affinity-purified anti-hDUS2 polyclonal antibodies; hDUS2 protein was localized in the cytoplasm of A549 cells. Subsequently, we stained A549 cells using both anti-hDUS2 antibodies and ER-Tracker (Molecular Probes, Eugene, OR) and confirmed its localization mainly at endoplasmic reticulum (Fig. 1D).

**Inhibition of growth of non–small cell lung cancer cells by specific small interfering RNA against hDUS2.** To assess whether hDUS2 is essential for growth or survival of lung cancer cells, we constructed plasmids to express siRNAs against hDUS2 (si-hDUS2) as well as control plasmids (siRNAs for Scramble and Luciferase, or mock vector) and transfected them into cell lines A549 and LC319. The protein levels in cells transfected with si-hDUS2-#2 were significantly decreased in comparison with cells transfected with either control siRNAs or si-hDUS2-#1. We observed significant decreases in the number of colonies formed and in the numbers of viable cells measured by MTT assay (representative data of A549 was shown in Fig. 3A).

**Identification of EPRS as a protein interacting with hDUS2.** To elucidate the function of hDUS2 in lung cancer cells, we looked for protein(s) that would interact with it. Lysates of LC319 cells transfected with pCAGGS-n3FH-hDUS2 vector or mock vector (control) were extracted and immunoprecipitated with anti-FLAG M2 monoclonal antibody followed by immunoprecipitation with anti-HA M2 monoclonal antibody. Protein complexes were stained with SilverQuest (Invitrogen) on SDS-PAGE gels. A 180-kDa band, which was seen in immunoprecipitates of cell lysates transfected with hDUS2-expressing plasmids but not in those with mock plasmids, was extracted and its peptide sequence was determined by MALDI-TOF-MS. This procedure identified EPRS as a candidate for interaction with hDUS2. We confirmed the cognate interaction between endogenous hDUS2 and exogenous EPRS, by immunoprecipitation experiment (Fig. 3B). Immunocytochemical analysis using LC319 cells transfected with myc-tagged-hDUS2 (pcDNA-hDUS2) and FLAG-tagged-EPRS
(pCMV10-EPRS) revealed colocalization of the proteins (Fig. 3C), suggesting that an hDUS2-aminoacyl tRNA synthetase complex may be required for translation to occur.

**tRNA-dihydrouridine synthase activity of hDUS2 in human cells.** To test our hypothesis that hDUS2 encoded a member of the DUS family, we investigated the effects of hDUS2 expression or suppression on levels of tRNA-dihydrouridine. Using HPLC, we purified tRNA from total RNA of A549 cells transfected either with plasmids expressing hDUS2 (pCAGGS-n3FC-hDUS2) or mock vector (control). We also purified that from the cells transfected either with siRNA constructs against hDUS2 or Scramble (control). We then measured the dihydrouridine content in each of the transfected cultures. Levels of dihydrouridine in tRNA from cells transfected with the hDUS2 expression vector were significantly increased compared with cells containing control vector (Fig. 4A). On the other hand, the most effective hDUS2 siRNA construct (si-hDUS2-#2), which had suppressed levels of protein in other experiments, caused a reduction of the tRNA-dihydrouridine content compared with the control siRNA construct (si-Scramble; Fig. 4B). The significant reduction of tRNA-dihydrouridine induced by si-hDUS2-#2 in these cells were closely related to suppression of tumor cell growth (see Fig. 3A), implying that tRNA-DUS activity is critical for survival of lung cancer cells. We then tested the effect of hDUS2 on in vitro translation of poly(A) RNA, using a rabbit-reticulocyte lysate system. Addition of GST-hDUS2 recombinant protein to a mixture of the lysate and poly(A) RNA from A549 cells increased the amount of translated protein on SDS-PAGE analysis in a dose-dependent manner (Fig. 4C), implying that hDUS2 is a significant factor in positive regulation of protein synthesis and confers a rapid growth advantage on cancer cells.

**Association of hDUS2 overexpression with poor prognosis.** Using tissue microarrays prepared from 298 NSCLCs, we did immunohistochemical analysis with affinity-purified anti-hDUS2 polyclonal antibodies and found positive staining in 263 (88%) cases. Of those, 135 were adenocarcinoma tumors (85% of 158); 89 were SCCs (90% of 99 cases); 19 were LCCs (90% of 21 cases); all

![Figure 5](https://example.com/figure5.png)
10 bronchioloalveolar cell carcinoma and all 10 adenosquamous carcinoma cases (100%) stained positively as well. All of these tumors were surgically resected NSCLCs, and no staining was observed in any of their adjacent normal lung tissues (Fig. 5A).

We classified patterns of hDUS2 expression as absent/weak (scored as 0 to 1+) or strong (scored as 2+). We then tried to correlate expression of this protein with various clinicopathologic variables. Statistical analysis revealed no significant correlation with pT/C0 or pN/C0 factors, although the Kaplan-Meier method indicated significant association between strong staining in NSCLCs and tumor-specific 5-year survival (P = 0.0019 by the log-rank test; Fig. 5B). By univariate analysis, pT (T1 versus T2-4), pN (N0 versus N1-2), age (>64 versus ≤64), and hDUS2 expression (0, 1+ versus 2+) were all significantly related to poor tumor-specific survival among NSCLC patients (P = 0.0002, <0.0001, 0.0129, and 0.0022, respectively). Furthermore, multivariate analysis using the Cox proportional-hazard model indicated that hDUS2 staining was an independent prognostic factor for NSCLC (P = 0.0013).

Discussion

Although advances have been made in development of molecular-targeting drugs for cancer therapy, the proportion of patients showing good response to available treatments is still very limited (16). Hence, it is urgent to develop new anticancer agents that will be highly specific to malignant cells, with minimal or no adverse reactions. A powerful strategy in this direction could be achieved by (a) screening for up-regulated genes in cancer cells based on genetic information obtained on cDNA microarrays, combined with high-throughput screening of their effects on cell growth; (b) investigation of loss-of-function phenotypes with RNAi systems and determination of biological functions of the proteins; and (c) systematic analysis of protein expression among hundreds of clinical samples on tissue microarrays. Using these approaches, we have shown here that hDUS2, a novel human homologue of yeast and bacterial DUS enzymes, is frequently overexpressed in clinical lung cancer samples and NSCLC cell lines, and that the gene product is necessary for survival/growth of lung cancer cells.

To date, >40 different types of tRNA modification have been described in eukaryotes (17). Modification of tRNA in response to various drugs occurs at very different levels from one organ to another, in cells at different differentiation levels or under different physiologic conditions such as aging or starvation and also in malignant cells (18). High levels of tRNA methylase activity in tumor cells seem to cause hypermethylation of tRNA (19), suggesting that hypermodified tRNAs may have a specific functional role in carcinogenesis. An increase in the level of dihydrouridine was found in tRNA_phe purified from human malignant tissues nearly three decades ago (12). Dihydrouridine is one of the most frequent modifications observed in nucleosides of tRNA from bacteria to eukaryotes and is found at multiple different positions in tRNA; most of them are present in the D-loop (20, 21). Most of tRNA modification enhances structural stabilization of tRNA (22). However, dihydrouridine is assumed to increase conformational flexibility and dynamic motion of the tRNA (10) and to confer a growth advantage on organisms such as psychrophilic bacteria, under conditions where the dynamics of various environmental factors are severely compromised (11). DUS enzymes have been identified in S. cerevisiae and E. coli (9, 21, 23), but no DUS enzymes with DSRM were previously identified in humans.

To elucidate the biological function of hDUS2 and its contribution to lung carcinogenesis, using siRNA technology we suppressed hDUS2 in a human NSCLC cell line, A549, in which the gene is highly expressed endogenously. Suppression of endogenous hDUS2 reduced tRNA-DUS activity and suppressed growth of A549 cells. In addition, we showed by means of an in vitro rabbit-reticulocyte lysate system that hDUS2 protein was likely to increase the efficiency of translation. On the other hand, when
we detected by semiquantitative RT-PCR mRNA expression of hDUS1, which encodes other DUS enzyme and possibly corresponds to one unannotated transcript (Homo sapiens PP3111 protein, accession no. NM_022156), no differential expression of hDUS1 between tumor and adjacent normal lung tissue was detectable in most of samples we examined (data not shown). The result suggested that activation of specific DUS enzyme(s) like hDUS2 could play a significant role for growth of lung cancer cells.

We also documented interaction of hDUS2 with a multifunctional aminoacyl-tRNA synthetase, EPRS, which catalyzes the aminoacylation of individual tRNA species (24). This is an essential first step in translation of a genetic message because, together with codon-anticodon recognition, the specificity of this reaction determines the fidelity of mRNA translation. In vivo and in vitro experiments have revealed that dihydrouridine 20 (D20) is a key identity element for interaction with the β-subunit of phenylalanyl-tRNA synthetase (PheRS; ref. 25). The combined evidence suggests that subtle structural perturbations may affect formation of the tRNA+PheRS complex. Our data suggest that conformational flexibility of the tRNA conferred by hDUS2 might be integral to the recognition of D-loop identity elements for aminoacyl-tRNA synthetases, promote specific aminoacylation in human cells, and enhance the translation process in cancer cells (Fig. 6).

Clinicopathologic evidence obtained through our tissue microarray experiments showed that NSCLC patients with strongly hDUS2-positive tumors showed shorter cancer-specific survival periods than those with negative or weak staining for hDUS2, raising the possibility that overexpressed hDUS2 could signal a highly malignant phenotype of lung cancer cells.

To our knowledge, this is the first report to indicate that levels of hypermodified tRNAs, such as tRNA-dihydrouridylate, have a specific functional role for growth and/or survival of cancer cells, through the agency of a novel tRNA-DUS protein complex with aminoacyl tRNA synthetase. These data strongly imply the possibility of designing new anticancer drugs to specifically target the enzymic activity of hDUS2 and/or the hDUS2-tRNA synthetase complex, as a promising therapeutic and diagnostic strategy for treatment of lung cancer patients.

Acknowledgments

Received 2/21/2005; revised 4/16/2005; accepted 4/21/2005.

Grant support: The Japan Society for the Promotion of Science “Research for the Future” Program 08104102 (Y. Nakamura).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

A Novel Human tRNA-Dihydouridine Synthase Involved in Pulmonary Carcinogenesis

Tatsuya Kato, Yataro Daigo, Satoshi Hayama, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/13/5638

Cited articles
This article cites 24 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/13/5638.full.html#ref-list-1

Citing articles
This article has been cited by 31 HighWire-hosted articles. Access the articles at:
/content/65/13/5638.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.