Clinicopathological and prognostic significance of nuclear UGDH localization in lung adenocarcinoma

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ABSTRACT

This study aimed to clarify relationships among UDP-glucose-6 dehydrogenase (UGDH) expression, clinicopathological factors, and the prognosis of patients, and to determine the role of UGDH in lung adenocarcinoma (AC). Firstly, UGDH expression and localization in 126 lung AC tissues were immunohistochemically studied, and associations with clinicopathological parameters and patients’ prognosis were evaluated. Secondly, serum UGDH levels were measured in 267 lung cancer patients and 100 healthy controls. Finally, the effects of UGDH knockdown by siRNA on migration and invasion abilities were analyzed. As a result, nuclear UGDH staining was significantly correlated with poorer differentiation, a larger tumor size, higher p-TNM stage, positive nodal metastasis, positive lymphatic invasion, and positive vascular invasion in lung AC patients. Nuclear UGDH-positive patients showed significantly poorer survival than nuclear UGDH-negative patients. Serum UGDH levels were especially higher in lung AC patients even in stage I than those in healthy controls. In lung AC cell lines, nuclear expression levels of UGDH were higher in LC-2/ad cells than in A549 cells. UGDH siRNA-treated LC-2/ad cells showed significantly decreased migration and invasion abilities, but no significant differences were observed in UGDH siRNA-treated A549 cells. These data indicate that UGDH expression and localization are an early sero-diagnostic marker in addition to a poor prognostic indicator in lung AC patients.

Lung cancer is a leading cause of cancer-related death worldwide. Non-small cell lung carcinoma (NSCLC) is a major subtype in lung cancer. In a previous report, about 40% of NSCLC patients were diagnosed as stage III/IV at the first visit to a doctor in Japan (23). Five-year survival rates of patients with resectable NSCLCs are about 80% for stage I/II, but the rates decrease to about 40% for stage III/IV (28). For the identification of patients at an early stage, novel tools including biomarkers to diagnose early-stage lung cancer are expected.

UDP-glucose-6 dehydrogenase (UGDH) is a metabolic enzyme that converts UDP-glucose to UDP-glucuronic acid. UDP-glucuronic acid is a known substrate for glucuronidation and the synthesis of glycosaminoglycans including hyaluronic acid (HA), chondroitin sulfate (CS), and heparan sulfate (HS). Several studies showed that UGDH expression is up-regulated by TGF-β and downregulated by hypoxia, and that factors regulating UGDH expression are associated with tumor progression (4, 7, 9). This evidence indicates the possibility that a change of
UGDH expression leads to tumor progression. Recently, Li et al. reported that accumulations of HA and collagen were associated with a high tumor interstitial pressure, vascular collapse, and hypoxia in tumor cells (22). In addition, up-regulation of UGDH was observed in drug-resistant cell lines (19, 26). However, the precise role of UGDH in the cancer microenvironment is not sufficiently understood.

Aiming to evaluate the role of UGDH in lung cancer, we analyzed UGDH localization in lung adenocarcinoma (AC) tissues and its expression levels in patients’ sera. Moreover, we investigated the effects of UGDH on the migration and invasion behaviors of lung AC cell lines.

In this study, we firstly identified a significant correlation between the nuclear localization of UGDH and various clinicopathologic parameters or the prognosis of patients with lung AC. We also clarified that serum UGDH expression levels were significantly higher in lung cancer patients, especially with AC, than in healthy controls.

MATERIALS AND METHODS

Lung AC cell lines. A549 was purchased from the American Type Culture Collection (Rockville, MD, USA). LC-2/ad was purchased from the RIKEN BioResource Center (Ibaraki, Japan). The cell lines were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (MP Biomedicals, Inc., Santa Ana, CA, USA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO₂ and 95% humidified air. Sub-confluent cells were harvested and washed 3 times with phosphate-buffered saline without divalent ions (PBS (−)), and fixed in 10% formalin or stored at −80°C.

Antibody. Ku-Lu-20 mouse monoclonal antibody, which was immunized with A549 cell lysates and recognized UGDH (Supporting Fig. 1), was generated in our laboratory (Supporting data). Anti-GAPDH mouse monoclonal antibody (clone 2D4A7; Abnova, Taipei, Taiwan) was used as an internal control. Anti-heat shock protein (HSP) 90 mouse monoclonal (clone F-2; Santa Cruz, Dallas, TX, USA) and anti-Histone H1 mouse monoclonal (clone AE-4, Abnova) antibodies were used as cytoplasmic and nuclear fraction markers, respectively. Anti-Akt (clone C67E7), anti-Akt-pS473 (clone D9E) rabbit monoclonal antibodies, anti-MAPK, and anti-MAPK-pT202/Y204 rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and used to analyze signal pathways.

Patients’ specimens. Ten percent formalin-fixed and paraffin-embedded tissues obtained from 126 lung AC patients who underwent surgical resection from January 2003 to December 2012 at Kitasato University Hospital were used in this study. Various clinical and pathological parameters were reviewed and analyzed according to the 7th edition of the TNM classification (13) (Table 1). The 126 ACs consisted of 45 lepidic (35.7%), 16 acinar (12.7%), 41 papillary (32.5%), 9 micropapillary (7.2%), 12 solid (9.5%) and 3 invasive mucinous (2.4%) adenocarcinomas according to the 2015 WHO classification (14). Sera from 100 healthy control and 267 patients with lung cancer were used for reverse-phase protein array (RPPA) analysis (Table 2). This study was approved by the Ethics Committee of the Kitasato University School of Medicine (B7-06) and followed the Declaration of Helsinki protocol. All patients were approached based on approved ethical guidelines, agreed to participate in this study, and could refuse entry and discontinue participation at any time. All participants provided written consent.

UGDH immunostaining in lung AC tissues and cell lines, and statistical analysis. Three-micrometer-thick sections of formalin-fixed and paraffin-embedded lung AC tissues and cell lines were deparaffinized in xylene and rehydrated in a descending ethanol series. After endogenous peroxidase treatment with 3% hydrogen peroxide for 10 min, the sections were antigen-retrieved in 0.01 M sodium citrate buffer (pH 6.0) with 0.1% Tween 20 at 121°C for 10 min by autoclaving. After cooling to room temperature (RT) and blocking with 0.5% casein for 10 min, the sections were incubated with undiluted mouse anti-UGDH antibody (hybridoma supernatant) overnight at RT. Then, the sections were reacted with EnVision (Dako) for 30 min at RT, and visualized with Stable DAB solution (Invitrogen, Carlsbad, CA, USA) for 5 min. During each incubation and reaction step, the sections were washed three times with Tris (hydroxymethyl) aminomethane-buffered saline (TBS) for 5 min each.

For UGDH, nuclear or cytoplasmic staining of tumor cells was considered to be positive. The stainability of bronchial epithelial cells was used as an internal positive control. The staining intensity was categorized into four groups by comparing the staining intensity of tumor cells with bronchial epithelial
cells: negative = no positive cells were observed; weak = weaker than epithelial cells; moderate = the same as epithelial cells; strong = stronger than epithelial cells. A result of more than 25% of tumor cells with moderate to strong cytoplasmic or nuclear staining was judged as positive.

Relationships between UGDH staining and clinicopathological factors were assessed by the \( \chi^2 \) test. The cumulative survival of patients was estimated using the Kaplan-Meier method, and the significance of differences in survival between UGDH-positive and -negative groups separately for nuclear and cytoplasmic staining was tested using the log-rank test. The Cox hazards regression model was used for multivariate analysis of prognostic factors. Differences were considered significant with a \( P \)-value less than 0.05. All reported \( P \)-values are two-sided.

Statistical analyses were performed using Stat flex statistical software package version 5.0 (Artech Co., Ltd., Osaka, Japan).

**Sub-cellular localization of UGDH in AC cell lines.** To confirm the nuclear localization of UGDH, we extracted and separated protein from A549 and LC-2/ad cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), and cytoplasmic and nuclear proteins were collected. For Western blotting, UGDH in 3 μg of each fraction was used. Anti-HSP 90 antibody (Santa Cruz) and anti-Histone H1 antibody (Abnova) were used as specific cytoplasmic and nuclear protein markers, respectively.

**Western blotting.** Proteins were extracted from lung...
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Slide for dimethyl sulfoxide (SDM 0011; Matsunami glass Inc., Osaka, Japan) in quadruplicate with a 640-spot/slide format using Glass Slide Microarrayer (V&P Scientific Inc., San Diego, CA, USA). Finally, the slides were scanned on a microarray scanner GenePix 4000b (Molecular Devices, Sunnyvale, CA, USA) and analyzed by GenePix pro 6.0 software package (Molecular Devices). $P < 0.05$ was considered significant.

Transfection of UGDH siRNA, and migration and invasion assay. For siRNA transfection, four sequences of UGDH siRNA (Qiagen, Venlo, Netherlands) and negative control siRNA (Takara Bio Inc., Shiga, Japan) were used. A total of $1 \times 10^7$ LC-2/ad cells were cultured in a 24-well plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After 24 h, cells were transfected with Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). Final concentrations of 250 nM of each siRNA for the UGDH gene and negative control were used. For migration and invasion assays, cells, resuspended at $1 \times 10^4$ cells per 0.3 mL in serum-free RPMI-1640 medium, were added to the upper chambers of 24-well Transwell inserts (Corning, Corning, NY, USA) coated with 50 μL of Matrigel (Corning) dissolved in serum-free RPMI-1640 medium for the invasion assay or non-coated ones for the migration assay (21). Bottoms of the wells in both assays were filled with 0.6 mL of RPMI-1640 medium supplemented with 10% FBS. After 24 h culture, cell culture inserts were removed, the upper surfaces of the filters were cleaned thoroughly with cotton swabs, and the lower surfaces were fixed for 10 min with 4% paraformaldehyde. Subsequently, the cells on the lower surfaces were visualized by hematoxylin and eosin staining. Cells in three random high-power fields were counted in triplicate. Student's $t$-test was used for statistical analysis.

RESULTS

UGDH staining in lung AC tissues and statistical analysis

The clinicopathological characteristics of the 126 patients are summarized in Table 1. A total of 59 males and 67 females were included in this study, and their ages ranged from 37 to 81 years (median, 64 years), of which 69 (54.8%) were smokers and 63 (50.0%) had stage I disease. A total of 70 patients were alive at the end of the follow-up period, 37 patients died of lung cancer, and 19 patients died from other causes. To evaluate the relationships be-
UGDH in lung adenocarcinoma

Expression of UGDH in normal lung tissues and lung adenocarcinomas. (A) No obvious staining was observed in normal alveolar epithelial cells. (B) Weak to moderate expression of UGDH was observed in the cytoplasm and/or nucleus of normal bronchial epithelial cells. Strong UGDH staining was observed in the nucleus (C) and cytoplasm (D) of lung adenocarcinoma cells (original magnification: A, B, C, D ×200).

All 126 patients were included in the cumulative survival analysis, with an overall follow-up period from 4.4 to 126.7 months (median, 83.9 months). The Kaplan-Meier survival curve was used to evaluate the correlation between UGDH and the prognosis of lung AC patients (Fig. 2). The analysis showed that nuclear UGDH-positive patients have a poorer prognosis than negative patients ($P = 0.0011$). The five-year cumulative survival probability was 67.7% for nuclear UGDH-positive patients vs. 89.0% for nuclear UGDH-negative patients. In contrast, no significant difference was observed in cytoplasmic UGDH staining ($P = 0.082$). The five-year cumulative survival probability was 72.8% for cytoplasmic UGDH-positive patients vs. 84.1% for cytoplasmic UGDH-negative patients. Univariate analysis was performed according to the Cox proportion hazard model to evaluate the effects of UGDH expression and other clinicopathological factors on survival (Table 3). In the univariate analysis, the crude haz-
card ratio (HR) for nuclear UGDH-positive patients compared with nuclear UGDH-negative patients was 2.08 (95% CI 1.10–3.93; \( P = 0.024 \)), indicating that nuclear UGDH-positive patients had a 2-times higher risk of cancer-specific mortality compared with nuclear UGDH-negative patients. No correlation was

Table 3  Uni- and multi-variate analyses of effect of UGDH localization on survival

| Factors                          | Univariate Analysis | Multivariate Analysis |
|----------------------------------|---------------------|-----------------------|
|                                  | HR      | 95% CI      | P-value | HR      | 95% CI      | P-value |
| Nuclear UGDH staining            |         |             |         |         |             |         |
| Positive vs. Negative            | 2.08    | 1.10–3.93   | \( 0.024 \) | 0.95    | 0.34–2.63   | 0.923   |
| Cytoplasmic UGDH staining        |         |             |         |         |             |         |
| Positive vs. Negative            | 1.60    | 0.86–2.97   | 0.138   | 2.19    | 0.92–5.19   | 0.076   |
| Age                              |         |             |         |         |             |         |
| \( \geq 65 \) vs. <65            | 1.05    | 0.58–1.90   | 0.869   | –       | –           | –       |
| Sex                              |         |             |         |         |             |         |
| Male vs. Female                  | 1.04    | 0.58–1.87   | 0.898   | –       | –           | –       |
| Smoking habits                   |         |             |         |         |             |         |
| Smoker vs. Never smoker          | 1.74    | 0.95–3.16   | 0.071   | –       | –           | –       |
| Tumor differentiation            |         |             |         |         |             |         |
| Moderately/Poorly vs. Well       | 2.73    | 1.47–5.05   | \( 0.001 \) | 1.60    | 0.62–4.15   | 0.334   |
| Tumor size                       |         |             |         |         |             |         |
| \( \geq 3 \) cm vs. <3 cm        | 2.28    | 1.24–4.18   | \( 0.008 \) | 1.15    | 0.43–3.07   | 0.788   |
| p-TNM stage                      |         |             |         |         |             |         |
| Stage II/III vs. Stage I         | 3.43    | 1.87–6.30   | \( < 0.001 \) | 1.04    | 0.28–3.84   | 0.949   |
| Nodal status                     |         |             |         |         |             |         |
| N1, N2, N3 vs. N0                | 3.58    | 1.98–6.48   | \( < 0.001 \) | 0.83    | 0.23–3.00   | 0.782   |
| Pleural invasion                 |         |             |         |         |             |         |
| Yes vs. No                       | 2.16    | 1.18–3.95   | \( 0.012 \) | 1.39    | 0.57–3.43   | 0.471   |
| Lymphatic invasion               |         |             |         |         |             |         |
| Yes vs. No                       | 4.72    | 2.41–9.22   | \( < 0.001 \) | 3.25    | 1.10–9.62   | \( 0.033 \) |
| Vascular invasion                |         |             |         |         |             |         |
| Yes vs. No                       | 3.97    | 2.06–7.63   | \( < 0.001 \) | 1.98    | 0.68–5.75   | 0.207   |

Fig. 2  Kaplan-Meier curves show overall survival of patients with lung adenocarcinoma. Nuclear UGDH-positivity was significantly correlated with poorer survival compared with nuclear UGDH-negativity (\( P = 0.0011 \)). There was no significant correlation between cytoplasmic UGDH staining and patients' prognosis (\( P = 0.082 \)).
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and healthy controls were analyzed by the RPPA method. Serum UGDH levels from patients with lung cancer were significantly higher than in healthy controls ($P < 0.01$). Serum UGDH levels were also higher in AC patients than in healthy controls ($P < 0.01$) (Fig. 4A). Serum UGDH levels significantly increased simultaneously with tumor progression ($P < 0.01$) (Fig. 4B).

Transfection of UGDH siRNA, and migration and invasion assay
Nuclear UGDH-positive tumors were closely associated with pleural, lymphatic, and vascular invasion (Table 1), and nuclear expression levels of UGDH were markedly different in LC-2/ad and A549 cells (Fig. 4). To investigate whether UGDH served different roles depending on its sub-cellular localization, transwell assays with both cells treated with UGDH siRNA were performed. In four UGDH

Sub-cellular localization of UGDH in AC cell lines
In IHC, UGDH staining was observed in the nucleus in addition to the cytoplasm. The stainability of nuclear UGDH was stronger in LC-2/ad cells than in A549 cells (Fig. 3A). However, few studies have reported the nuclear localization of UGDH. To confirm the nuclear localization of UGDH, fractionated proteins from both lung AC cell lines were analyzed by Western blotting (Fig. 3B). UGDH expression was detected in both the nuclear and cytoplasmic fractions. Nuclear expression levels were higher in LC-2/ad cells than in A549 cells, consistent with the results of IHC.

Serum UGDH levels
UGDH levels in sera from patients with lung cancer and healthy controls were analyzed by the RPPA method. Serum UGDH levels from patients with lung cancer were significantly higher than in healthy controls ($P < 0.01$). Serum UGDH levels were also higher in AC patients than in healthy controls ($P < 0.01$) (Fig. 4A). Serum UGDH levels significantly increased simultaneously with tumor progression ($P < 0.01$) (Fig. 4B).
ties (29). UGDH expression is one of the factors that controls the production of glycosaminoglycans as important components of ECM (5). Therefore, UGDH has been considered to be involved in the invasion ability and drug resistance via the biosynthesis of glycosaminoglycans such as HA in tumor tissues. A recent report showed that UGDH was significantly correlated with migration in colorectal carcinoma (34) and glioblastoma (24). However, it is insufficient to fully understand the role of UGDH in tumor tissues. There are a few reports on mRNA expression of UGDH levels in tumors; however, no studies have evaluated the relationship between UGDH protein expression levels and clinicopathological factors in tumors. To our knowledge, the localization of UGDH in tumor cells has also not been reported. In this study, we clarified that nuclear UGDH staining was significantly correlated with clinicopathological parameters including vascular invasion, lymphatic invasion, lymph node metastasis, and the prognosis of the patients with lung AC. We also found that UGDH localized in both the nucleus and cytoplasm of lung AC cells and tissues. According to the Reactome database (10), cytoplasmic UGDH is involved in converting UDP-glucose. However, the function of nuclear UGDH has not been clarified. The present IHC study indicated that nuclear UGDH staining was significantly correlated with clinicopathological parameters including vascular invasion, lymphatic invasion, lymph node metastasis, and the prognosis of the patients with lung AC.

**DISCUSSION**

Glycosaminoglycan is a major component of the extracellular matrix (ECM). HA, CS, and HS are well-known as important components regulating cancer events such as invasion and metastasis (3, 22). Altered production of ECM components in tumors has been reported (33). Tumors with excess ECM components have high cell migration and invasion abilities (29). UGDH expression is one of the factors that controls the production of glycosaminoglycans as important components of ECM (5). Therefore, UGDH has been considered to be involved in the invasion ability and drug resistance via the biosynthesis of glycosaminoglycans such as HA in tumor tissues. A recent report showed that UGDH was significantly correlated with migration in colorectal carcinoma (34) and glioblastoma (24). However, it is insufficient to fully understand the role of UGDH in tumor tissues. There are a few reports on mRNA expression of UGDH levels in tumors; however, no studies have evaluated the relationship between UGDH protein expression levels and clinicopathological factors in tumors. To our knowledge, the localization of UGDH in tumor cells has also not been reported. In this study, we clarified that nuclear UGDH staining was significantly correlated with clinicopathological parameters including vascular invasion, lymphatic invasion, lymph node metastasis, and the prognosis of the patients with lung AC.

Because nuclear UGDH expression was linked to tumor cell invasion, we compared expression levels of invasion-associated pathway markers between UGDH knockdown LC-2/ad cells and control cells. Western blot analysis revealed that the pAkt level was positively regulated by UGDH expression. However, no correlation between UGDH and pMAPK was observed (Fig. 5D). These data suggest the possibility that nuclear UGDH participates in tumor cell migration and invasion of lung AC via Akt signaling pathways.

**siRNAs, siRNA-#1 most effectively knocked down the expression at protein levels. Thus, a later experiment used siRNA-#1 (Fig. 5A). UGDH knockdown LC-2/ad cells displayed decreased migration and invasion abilities, and cell numbers showed 55 and 72% reductions in comparison with siControl cells, respectively (both \( P < 0.05 \)) (Fig. 5B, C). On the other hand, no significant differences were observed in A549 cells.**
Fig. 5 The nuclear localization of UGDH controls migration and invasion. The effects of UGDH knockdown by siRNA were analyzed. (A) Western blot analysis of the effects of UGDH knockdown using four sequences of siRNAs in LC-2/ad cells. (B) Representative image (×100 magnification) and quantification of the transwell migration assay of LC-2/ad cells (left) and A549 cells (right). (C) Representative image (×100 magnification) and quantification of the transwell invasion assay of LC-2/ad cells (left) and A549 cells (right). Migrating and invading cells were stained with hematoxylin and eosin and counted under a microscope. All data are presented as the mean ± SD of at least three independent experiments. Significance was calculated by Student’s t-test. UGDH knockdown LC-2/ad cells significantly decreased migration and invasion abilities in comparison with siControl cells (both P < 0.05). No significant differences were observed in A549 cells. (D) Expression levels of UGDH, Akt, p-Akt, MAPK, and p-MAPK were analyzed by Western blot analysis in UGDH siRNA-treated LC-2/ad cells. UGDH positively regulated p-Akt expression levels but did not regulate p-MAPK expression levels. GAPDH was used as an internal control.
we considered that nuclear UGDH expression is more important for tumor progression than cytoplasmic UGDH expression.

Protein moonlighting is a phenomenon whereby a protein can perform more than one function (18). Ancestral moonlighting proteins originally possessed a single function but through evolution, acquired additional functions. The most common primary function of moonlighting proteins is enzymatic catalysis, but these enzymes have acquired secondary non-enzymatic roles. Some examples of functions of moonlighting proteins secondary to catalysis include signal transduction, transcriptional regulation, apoptosis, motility, and structural functions (31). Cytoplasmic enzymes, pyruvate kinase M2 (PKM2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), have acquired secondary non-enzymatic roles such as DNA repair protein in the nucleus and RNA-binding protein (12, 30), respectively, in addition to the primary enzymatic catalytic roles. These proteins with a glycolysis function have common characteristics to form homo-oligomer and their function and localization were known to differ based on their forms (2, 8, 25, 36). The wild-type UGDH forms homo-oligomers like moonlighting proteins (15, 16). In this study, we firstly clarified that its function markedly differed based on its sub-cellular localization, suggesting that UGDH might be a moonlighting protein. A functional study of nuclear UGDH is necessary to test this hypothesis.

We revealed that serum UGDH levels in AC patients even in the early stages significantly increased compared with those in healthy controls. In addition, we confirmed that up-regulated UGDH nuclear or cytoplasmic expression was observed in stage I ACs compared with non-neoplastic bronchial epithelial cells. A recent study showed that the serum HA level was correlated with the metastasis and aggressiveness of tumors (1, 35). There is no report on serum UGDH levels in cancer patients, but the expression levels may be elevated because overexpression of UGDH in tumor cells is detected.

In this UGDH siRNA knockdown study, we clarified that LC-2/ad cells with nuclear UGDH expression showed significantly decreased invasion and migration abilities compared with siRNA controls, but such a tendency was not observed in A549 cells with cytoplasmic UGDH expression. These functional assays also indicated that nuclear UGDH localization might be closely associated with invasion and migration of tumor cells. Recently, we reported similar results whereby the overall survival of lung AC patients differed in association with the sub-cellular localization of S100A16 in tumor cells (20, 27).

In the knockdown experiment, the suppression of UGDH expression was involved in decreasing phosphorylation levels of Akt in nuclear UGDH-positive LC-2/ad cells. Several reports showed that Akt signaling pathways were activated in many cancers, and Akt was a key protein involved in mediating various biological responses, such as the inhibition of apoptosis, cell survival, proliferation, and invasion (11, 17, 32). The actions of Akt are diverse and affect cells by phosphorylating a variety of downstream targets such as BAD, caspase-9, FKHR, GLUTs, mTOR, IKK, NF-kappa B, GSK3, p21cip1, CREB, p27kip1, Cjk1, and eNOS (6). Xue and Hemmings reported that hyperactivity of the Akt pathway promoted epithelial-mesenchymal transition (EMT) and metastasis due to its effects on cell migration (37). Since EMT plays a key role in the invasiveness of tumor cells, many of the proteins in Akt-related pathways could be targets of cancer therapeutics (39). Further analysis of up- and downstream molecules in Akt-related pathways involved in UGDH are needed.

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Production of monoclonal antibodies using the random immunization method. Fifty mg wet-weight of A549 cell lysate was prepared with PBS (−) as an immunogen using an ultra-sonic homogenizer (UH-50; SMT Company, Tokyo, Japan). Five-week-old female BALB/c mice were immunized intra-peritoneally with A549 cell lysate 3 times at two-week intervals. The antibody titer was tested by IHC using 100-times diluted sera from immunized mice as the first antibody on AMeX-fixed A549 cells. Three days prior to cell fusion, the mouse with the highest titer was intra-peritoneally boosted with the same amount of A549 lysate. Hybridoma production and hybridoma screening were conducted as previously described (Nagashio et al. (2010) Lung Cancer 69, 54–59). Random immunization combined with immunohistochemical screening can be used to select antibodies that react with targeted tumor cells at the time of screening and is useful to gain potential immunohistochemical antibodies. (Nitori et al. (2005) Clin Cancer Res 11, 2531–2539; Matsumoto et al. (2018) Pathol Int 68, 232–240)

Supporting Fig. 1 Identification of recognized antigen for KU-Lu-20 monoclonal antibody by immunoprecipitation and mass spectrometry. (A) The proteins, immunoprecipitated with KU-Lu-20 antibody, were separated with SDS-PAGE and the gel was stained with the Zn-staining kit [lane 1: A549 lysate combined with KU-Lu-20 antibody and protein G, lane 2: KU-Lu-20 antibody combined with protein G, lane 3: A549 lysate combined with protein G]. (B) Western blot analysis with immunoprecipitation samples and KU-Lu-20 hybridoma supernatant as the first antibody. Negative controls are lanes 4 and 5. Based on MALDI TOF/TOF-MS analysis, the KU-Lu-20 antibody recognized UGDH. [lane 1: A549 lysate, lane 2: Blank, lane 3: A549 lysate combined with KU-Lu-20 antibody and protein G, lane 4: KU-Lu-20 antibody combined with protein G, lane 5: A549 lysate combined with protein G]. (C) Confirmation of the identified antigen protein. KU-Lu-20 antibody reacted with the recombinant UGDH (ATGen, Seongnam, Korea), but not with the recombinant venus protein (a generous gift from Dr. Goshima, National Institute of Advanced Industrial Science and Technology). [lane 1: 0.3 μg of purified UGDH, lane 2: 0.3 μg of venus protein as a negative control].
Supporting Fig. 2  UGDH localization in lung adenocarcinoma was further confirmed using specimens from The Human Protein Atlas (www.proteinatlas.org). (Left image) Strong cytoplasmic staining was observed in tumor cells, while nuclear staining was not noted. (Right image) Nuclear and cytoplasmic staining was observed.