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Lipase member H is a novel secreted protein selectively upregulated in human lung adenocarcinomas and bronchioloalveolar carcinomas

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ABSTRACT

Lung cancer is one of the most frequent causes of cancer-related death worldwide. However, molecular markers for lung cancer have not been well established. To identify novel genes related to lung cancer development, we surveyed publicly available DNA microarray data on lung cancer tissues. We identified lipase member H (LIPH, also known as mPA-PLA1) as one of the significantly upregulated genes in lung adenocarcinoma. LIPH was expressed in several adenocarcinoma cell lines when they were analyzed by quantitative real-time polymerase chain reaction (qPCR), western blotting, and sandwich enzyme-linked immunosorbent assay (ELISA). Immunohistochemical analysis detected LIPH expression in most of the adenocarcinomas and bronchioloalveolar carcinomas tissue sections obtained from lung cancer patients. LIPH expression was also observed less frequently in the squamous lung cancer tissue samples. Furthermore, LIPH protein was upregulated in the serum of early- and late-phase lung cancer patients when they were analyzed by ELISA. Interestingly, high serum level of LIPH was correlated with better survival in early phase lung cancer patients after surgery. Thus, LIPH may be a novel molecular biomarker for lung cancer, especially for adenocarcinoma and bronchioloalveolar carcinoma.

1. Introduction

Lung cancer is one of the most frequent causes of cancer-related death worldwide in both men and women [1]. By histology, lung cancer is classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC is further classified into several major subtypes, such as adenocarcinoma (AC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). Among NSCLCs, AC is the most frequent subtype of lung cancer [2]. Many genetic alterations have been reported to be involved in the initiation and progression of lung cancer [3–6]. Moreover, recent studies using DNA microarrays demonstrated that gene expression patterns were distinct for lung cancers of different histological types [7,8]. However, molecular markers for various lung cancers have not yet been established.

In this study, we investigated novel biomarkers for lung cancer using a public microarray database. We identified lipase member H (LIPH) as one of the genes significantly upregulated in lung AC and bronchioloalveolar carcinoma (BAC). Moreover, we show that LIPH was associated with better survival in lung cancer patients.

2. Materials and methods

2.1. Cell culture

Sixteen NSCLC cell lines including 9 AC cell lines (PC-3, PC-14, RERF-LC-KJ, RERF-LC-MS, ABC-1, RERF-LC-Ad1, RERF-LC-Ad2, VMRC-LCD, and LC-2/Ad) and 7 squamous cell carcinoma (SCC) cell lines (PC-1, LC-1 sq, RERF-LC-AI, RERF-LC-Sq1, EBC-1, HARA, and LK-2) were used in this study. PC-1 and PC-14 cells were purchased from Immuno-Biological Laboratories (Fujioka, Japan). The human

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small airway epithelial cells (SAECs) and normal human bronchial epithelial cells (NHBECs) were purchased from Takara Bio (Ohtsu, Japan). Other cell lines were obtained from RIKEN BRC (Tsukuba, Japan) or JCRB Cell Bank (Osaka, Japan). ABC-1, EBC-1, PC-3, RERF-LC-MS, and VMRC-LCD cells were maintained in Eagle's minimum essential medium (EMEM, Wako [Osaka, Japan]) supplemented with 10% fetal bovine serum (FBS). HARA, LK-2, PC-1, PC-14, RERF-LC-Ad1, RERF-LC-Ad2, RERF-LC-AI, RERF-LC-KJ, and RERF-LC-Sq1 cells were maintained in RPMI 1640 medium (Wako) supplemented with 10% FBS. LC-1 sq cells were maintained in RPMI 1640/Ham's F-12 medium (Wako) with 10% FBS. LC-2/Ad cells were maintained in RPMI 1640/Ham's F-12 medium with 15% FBS. SAECs and NHBECs were maintained in small airway epithelial basal medium (SABM) and bronchial epithelial basal medium (BEBM) (Takara Bio), respectively. All cell culture media were supplemented with penicillin and streptomycin. All cells were maintained in a humidified incubator at 37 °C under an atmosphere of 5% CO_2.

2.2. Clinical samples from lung cancer patients

Early stage lung cancer tissue samples, tumor-adjacent normal tissues, and sera were obtained from 78 lung cancer patients at the University of Tokyo Hospital (see Table 2). Written informed consent was obtained from all patients enrolled in this study. Forty-four men and 34 women were included in the study. The median age of the patients was 68.0 years, and the range was 34–85 years. The patients were divided into six groups according to pathological subtypes: AC (51), BAC (10), SCC, (13), small cell lung cancer (SCLC, 2), adenosquamous cell carcinoma (1), and large cell neuroendocrine carcinoma (LCNEC, 1). Control serum samples were obtained from patients with non-cancerous lung disease such as epithelioid cell granuloma with necrosis, malignant mesothelioma, thymoma, atypical adenomatous hyperplasia, because of insufficient number of healthy volunteers. These frozen tissue samples were used for qPCR analysis (data not shown). The sera were used for ELISA as shown in Fig. 1G.

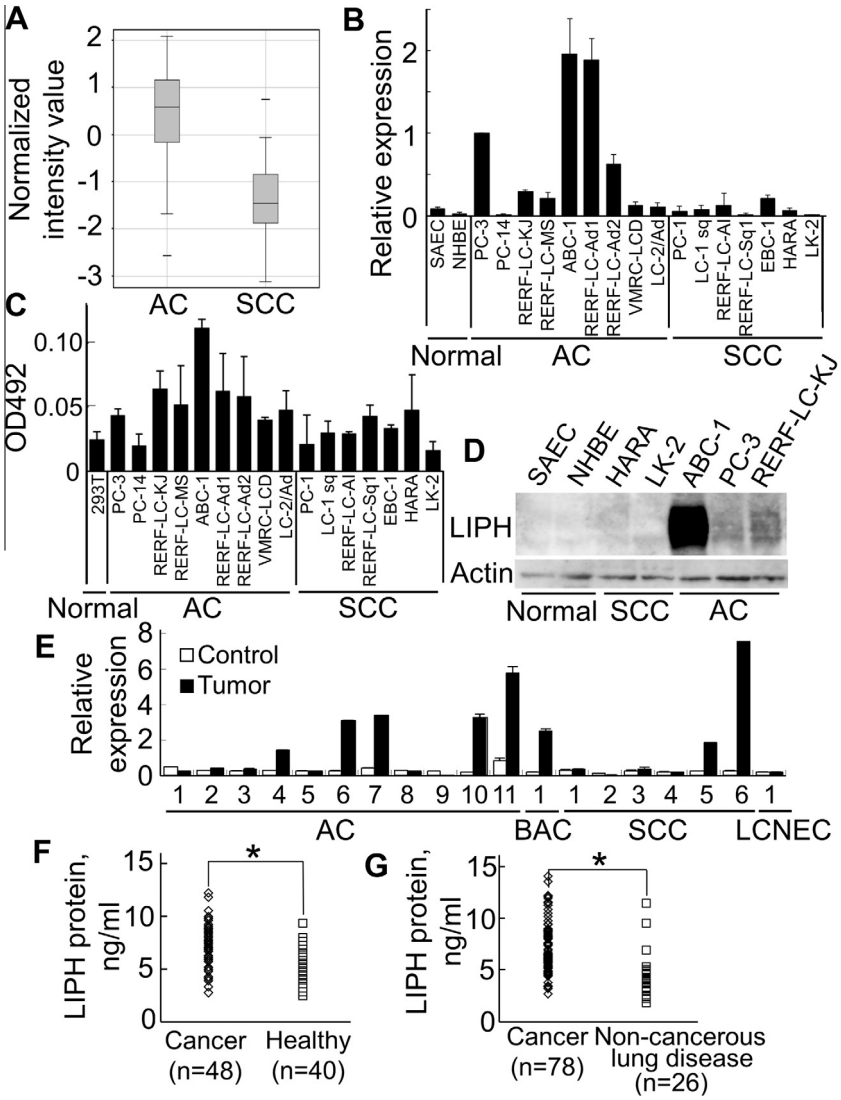

Fig. 1. Lipase member H (LIPH) is highly expressed in lung cancers. (A) Relative expression of the *LIPH* mRNA in adenocarcinoma (AC) and squamous cell carcinoma (SCC) analyzed using microarray data (GSE10245). AC, n=40; SCC, n=18. (B) *LIPH* mRNA expression in human lung cancer cell lines, normal small airway epithelial cells (SAECs), and bronchial epithelial cells (NHBEs) analyzed by qPCR. *LIPH* mRNA expression levels were measured by qPCR and normalized to *GAPDH* mRNA. The expression of *LIPH* in PC-3 cells was calculated as 1. (C) ELISA of LIPH protein secreted from AC and SCC cell lines. Lung cancer cell lines were cultured until 80% confluence. The samples for ELISA were collected after the cells were cultured in GIT medium for 72 h ($n = 2$). (D) Western blotting analysis of the LIPH protein in lung cancer cell lines and SAEC and NHBE cells. (E) *LIPH* mRNA expression in AC and tumor-adjacent non-cancerous tissue (control) samples from patients with pathological stages III or IV lung cancer. *LIPH* expression was measured by qPCR and normalized to that of *TBP* mRNA. ($n = 4$) (F and G) LIPH protein levels in the sera of patients. LIPH protein concentration in the sera of patients with lung cancer stages III and IV (F) and early stages (G) were measured by ELISA.

As for advanced stage samples, the stages III or IV lung cancer samples and the adjacent normal tissues obtained from 13 men and 6 women (median age 53.5 years, range of 42–70 years) and consisting of 11 ACs, 6 SCCs, 1 BAC, and 1 LCNEC were purchased from Bizcom, Japan (Tokyo, Japan). These frozen tissue samples were used for qPCR analysis as shown in Fig. 1E. All serum samples obtained from AC patients and healthy volunteers were purchased from Bizcom Japan. The sera were used for ELISA as shown in Fig. 1F. All tumors were classified by the pathologic TNM stage determined according to the International Union against Cancer guidelines. All experiments using human clinical samples obtained at the University of Tokyo Hospital were approved by the ethical committee at the University of Tokyo, and performed at the University of Tokyo. All experiments using commercially available human samples including human cancer cell lines were approved by the ethical committee at AIST and done at AIST.

2.3. Western blotting

Western blotting was performed as described previously [9]. Protein extracts (10 μg) were resolved by SDS–PAGE, transferred to PVDF membranes, and incubated with antibodies against LIPH (96602-AP, Proteintech, IL, USA), FLAG (M2, Sigma, MO, USA) and actin (Thermo Scientific, MA, USA). The protein bands were visualized using the LAS 1500 Image Analyzer (Fuji Film, Tokyo, Japan).

2.4. Enzyme-linked immunosorbent assay (ELISA)

For preparation of the conditioned medium, lung cancer cell lines were cultured on 6-well plates until they reached 80% confluence. Then, the medium was changed to serum-free culture medium GIT (Wako), and cells were further cultured for 72 h. The conditioned medium was collected and centrifuged at 1000 rpm for 5 min at room temperature. Sandwich ELISA plates were prepared by coating with 100 μg of LIPH rabbit polyclonal antibody (96602-AP, Proteintech) and blocking with 25% Block Ace (Nacalai tesque, Kyoto, Japan) overnight at 4 °C. Undiluted conditioned medium of lung cancer cell lines or serum samples diluted by 10-fold with 20% Block Ace in PBS were loaded onto the plates and incubated for 1 h at room temperature. After washing, another anti-LIPH mouse polyclonal antibody (HPA049079, Sigma) were added and incubated for 1 h at room temperature. The plates were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (IgG) for 1 h at room temperature. The signals were visualized with Ortho-Phenylenediamine (OPD), and the absorbance at 492 nm was measured using the Sunrise Rainbow plate reader (Tecan, Männedorf, Swiss). LIPH protein levels in the sera were calculated based on a standard curve generated using the recombinant LIPH protein (1.56–100 ng/mL) supplied in a commercial kit (Cusabio Biotech, Wuhan, China).

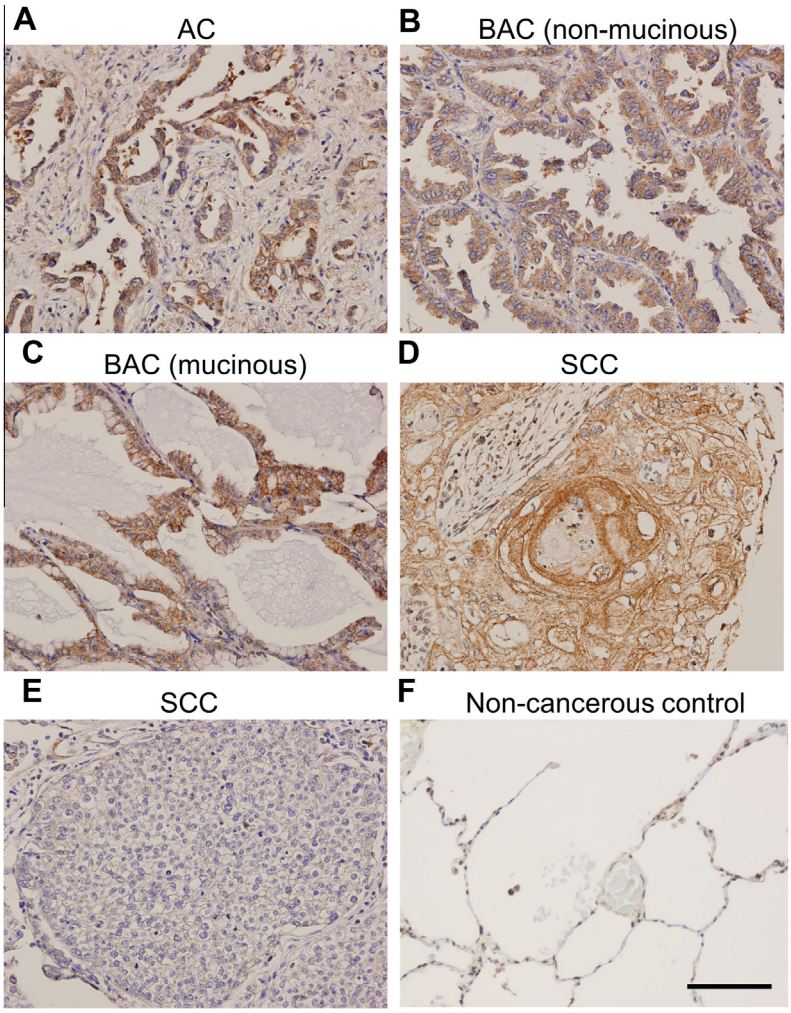

Fig. 2. Immunohistochemical analysis of LIPH in lung cancer tissue microarrays. LIPH expression in (A) AC, (B) non-mucinous BAC, (C) mucinous BAC, (D and E) SCC, and (F) non-cancerous control lung tissue samples assessed by immunohistochemistry. The images are representative of samples prepared as described in Section 2. Magnification, ×400. Size bar, 100 μm.

Table 1
Summary of immunohistochemical analysis of lung cancer tissue arrays.

Type of caner	Total cases	Strong positive[a]	Positive[b]	Merginal	Negative	a (%)	a + b (%)
AC	18	4	12	0	2	22	89
BAC	24	10	10	3	1	42	83
SCC	47	5	31	5	6	11	77
Ad-Sq	10	3	7	0	0	30	100
LCC	15	3	11	0	1	20	93
SCLC	10	1	3	3	3	10	40
Carcinosarcoma	2	0	2	0	0	0	100
Total cases	126	26	76	11	13		

Tissue arrays with 126 lung cancer tissue samples were immunostained with a LIPH antibody as shown in Fig. 2, and the immunopositive samples were counted. Percentage of (a) strong positive samples and (a+b) the sum of strong positive and positive samples were calculated and shown at the right side of the Table 1.

2.5. Immunohistochemistry

Lung cancer tissue microarray slides were purchased from Shanghai Outdo (Shanghai, china) and SuperBioChips (Seoul, South Korea). The tissue microarray slides containing 126 spots of formalin-fixed paraffin embedded tissue cores (18 ACs, 24 BACs, 47 SCCs, 10 adenosquamous cell carcinomas, 15 LCCs, 10 SCLCs, 2 carcinosarcomas), and the adjacent normal lung tissues for all cancer tissue samples were used for immunohistochemical analyses. The tissue microarray slides were soaked twice in xylene for 10 min for de-paraffinization. The slides were then treated sequentially with 100% ethanol for 10 min, 90% ethanol for 5 min, and 70% ethanol for 5 min, and rehydrated in deionized water for 10 min. Antigens were retrieved by boiling at 100 °C for 20 min in 10 mM citric acid buffer (pH 6.0). Staining was performed using the CSAII Biotin-free Tyramide Signal Amplification System (DAKO, Glostrup, Denmark) according to the manufacturer's instruction. A rabbit polyclonal antibody against LIPH (Proteintech, 16602-AP) was used at 1:100 dilution. The slides were counterstained with hematoxylin, and images were obtained using an Olympus IX59 microscope equipped with an EOS Kiss X6i camera (Canon, Tokyo, Japan).

2.6. Statistical analysis

Patients were divided into two groups according to their serum LIPH protein levels. The mean value of the serum LIPH protein level (6.0 ng/mL) calculated using data from all 78 patients was used as the threshold. Overall survival was determined as the duration from the day of surgery to the day of death resulting from all causes. Patients who were still alive at the end of the follow-up period were regarded as censored cases. To determine time-to-event outcomes, the lengths of time to a first event were compared using the log-rank test, whereas to estimate the absolute risk of each event for each group, the Kaplan–Meier method was used; $p < 0.05$ was considered to be statistically significant. All statistical analyses were performed using JMP statistical program Version 10.0 (SAS Institute, Cary, NC).

Detailed information about quantitative real-time RT-PCR, plasmid construction, RNA interference assay, wound-healing assay, and analysis of DNA microarray data can be found in Supplementary materials and methods.

3. Results

3.1. LIPH expression is upregulated in lung adenocarcinoma

To identify novel biomarker genes for lung cancer cells, we surveyed publicly available human lung cancer microarray data deposited in the NCBI GEO database. When gene expression in the lung AC and SCC was compared by bioinformatic analysis, 701 genes were identified as upregulated by more than 2-fold in AC compared to SCC (data not shown). Among these, we further selected genes encoding secretory proteins that could be detected in the sera of lung cancer patients. LIPH was identified as one of the secretory proteins significantly upregulated in the lung AC compared to SCC (Fig. 1A).

When LIPH expression was examined in various lung cancer cell lines, it was found that LIPH mRNA was upregulated in 6 out of 9 AC cell lines. In contrast, SCC cell lines expressed lower levels of LIPH (Fig. 1B). We further examined the LIPH protein secretion in the conditioned medium of lung cancer cell lines by the sandwich ELISA method. The highest levels of LIPH protein was observed in the ABC-1 conditioned medium followed by that of other AC cell lines, such as RERF-LC-kJ KJ and PC-3-KJ (Fig. 1C). Western blot analysis confirmed that LIPH protein of 55 kDa was secreted by these cells, but not by SCC cells or normal lung epithelial cells (Fig. 1D). These results indicate that LIPH is highly expressed in lung AC cell lines.

3.2. LIPH is upregulated in clinical samples from patients with advanced stage lung adenocarcinoma

Next, we analyzed *LIPH* mRNA expression in clinical samples from lung cancer patients by qPCR. *LIPH* mRNA was increased in tissues of advanced-stage lung cancer patients (8/19 cases, 42%).

Table 2
Characteristics of early stage lung-cancer patients used for prognostic analysis.

		LIPH ⩾ 6.0	LIPH < 6.0
		$n = 46$	$n = 32$
Gender	Male	23	21
	Female	23	11
Age	Median	67.7	68.2
	Standard deviation	8.4	11.0
Smoking status	Yes	28	18
	No	18	14
Clinical stage (TNM[a])	I	43	26
	II	0	2
	III–IV	3	4
Histology	AC[b]	37	23
	SCC	6	7
	Others	3	2
Pathological stage	I	35	22
	II	3	1
	III–IV	8	9
Surgical resection	R0	43	28
	R1	2	0
	R2	1	4
Operative procedure	Lobectomy	38	16
	Segmentectomy	1	5
	Wedge resection	6	10
	Others	1	1
Relapse	Yes	11	14
	No	35	18

[a] TNM, tumor, node, metastasis.
[b] AC includes BAC.

Moreover, in AC and BAC samples up-regulation of the *LIPH* mRNA was observed even more frequently (6/12 cases, 50%) (Fig. 1E). The LIPH protein levels in the sera of advanced-stage lung cancer patients were significantly higher compared to those in healthy individuals (Fig. 1F) and were also elevated in the sera of early-stage lung cancer patients compared to those with non-cancerous lung disease (Fig. 1G). However, qPCR analysis failed to show significant difference in *LIPH* expression for early stage lung cancer tissue samples (data not shown).

We further assessed the LIPH protein expression in various types of lung cancer samples by immunohistochemistry (Fig. 2 and Table 1). The LIPH-positive signal was clearly detected in cytoplasm of most of the ACs (16/18 cases, 88.9%) (Fig. 2A) and BACs (20/24 cases, 83.3%), including non-mucinous (Fig. 2B) and mucinous (Fig. 2C) BACs. Fewer SCCs samples were found to be LIPH-positive (36/47 cases, 76.6%) (Fig. 2D and E). The LIPH-positive signal was also detected in adenosquamous carcinoma and LCCs. Although some SCLCs expressed LIPH protein, their expression was heterogeneous and weak (data not shown). Control non-cancerous lung tissues showed limited and weak LIPH expression in type II alveolar epithelial cells and Clara cells (Fig. 2F). These results suggest that LIPH may be a consistent biomarker for NCSLC, especially AC and BAC.

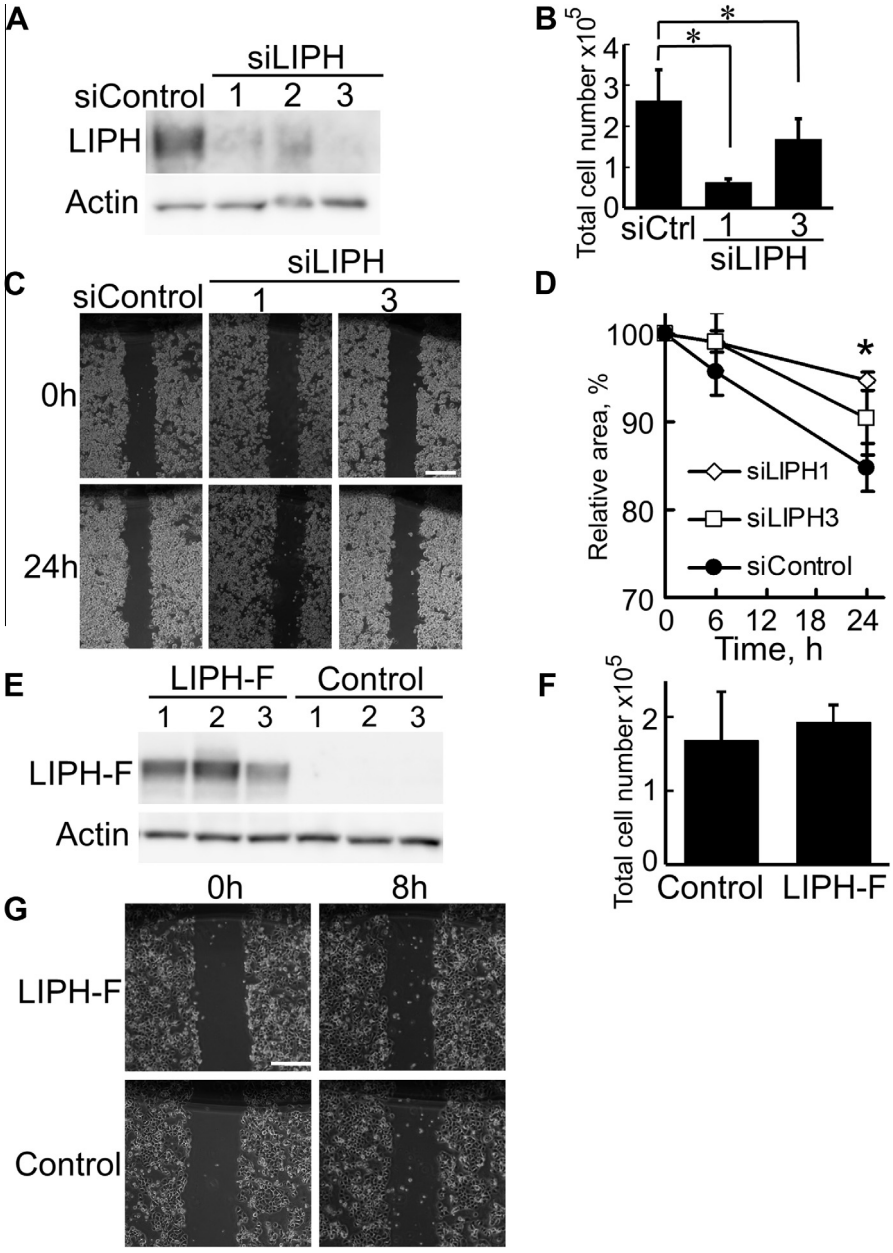

Fig. 3. LIPH is required for the proliferation of lung cancer cells. (A) Knockdown of endogenous LIPH in ABC-1 cells was evaluated by western blotting. Protein samples were extracted 72 h after transfection with 3 LIHP-specific siRNAs, and western blotting was performed using an anti-LIPH antibody. Actin was used as the loading control. (B) Cell proliferation of the LIPH siRNA1 or 3 (siLIPH1 or 3)-transfected ABC-1 cells. Total cell numbers were counted 72 h after transfection ($n = 6$, $^*p < 0.05$). (C) Wound-healing assay with siLIPH1 or 3-transfected ABC-1 cells. Images of the wound area were captured 0, 6, and 24 h after wound infliction and the representative images of 0h and 24h were shown. (D) The wound areas shown in (C) were measured using the Image J software ($n = 3$, $^*p < 0.05$). Size bar, 500 μm. (E) Western blotting analysis of LIPH ectopically expressed in RERF-LC-KJ cells with Flag antibody. Actin served as the loading control. (F) Proliferation of RERF-LC-KJ cells stably expressing LIPH-FLAG (LIPH-F). Cells were plated in 12-well plates ($n = 3$) and counted after 72 h. (G) Wound-healing assay with RERF-LC-KJ cells stably expressing LIPH-F. Cells were plated in 6-well plates and maintained until ⩾80% confluence. Images of cell monolayers were captured 0 and 8 h after wound infliction. The representative images were shown. Size bar, 500 μm.

3.3. LIPH promotes cell proliferation in lung cancer cells

Next, we investigated the biological functions of LIPH by knocking down or over-expressing the *LIPH* gene in lung cancer cell lines. When LIPH was knocked down in AC cells expressing high levels of endogenous LIPH (ABC-1) by transient transfection with LIPH-specific siRNAs (Fig. 3A), cell proliferation was markedly decreased (Fig. 3B). Moreover, the wound-healing assay revealed that LIPH knockdown suppressed wound closure (Fig. 3C and D), suggesting that endogenous LIPH contributes cell proliferation or cell migration into the wound area. Because cell migration was rarely observed with this cell line, we interpreted these results as an indication that endogenous LIPH is necessary for proliferation of lung cancer cells. Next, we established the AC cell line (RERF-LC-KJ) stably expressing LIPH with a C-terminal FLAG epitope (LIPH-F) (Fig. 3E). However, no significant difference was observed in cell proliferation and motility of the LIPH-over-expressing cells (Fig. 3F and G). Similar results were also obtained with the other LIPH-transfected AC cell lines, PC-14 and VMRC-LCD (data not shown).

3.4. Prognostic value of LIPH in lung cancer patients

We further investigated the prognostic relevance of LIPH expression in the lung cancer. Unexpectedly, overall survival in patients who had high serum LIPH protein levels (≥6.0 ng/mL, $n = 46$) was significantly better than that in patients with low serum LIPH protein levels (<6.0 ng/mL, $n = 32$) ($p = 0.0065$) (Fig. 4A). When we excluded 16 patients from the analysis who had received adjuvant chemotherapy, the overall survival in patients with high serum LIPH protein levels ($n = 37$) was once again better than that in patients with low serum LIPH protein levels ($n = 25$) ($p = 0.0035$) (Fig. 4B). These results suggest that high LIPH levels in sera were correlated with better survival in early phase lung-cancer patients after surgery. Thus, LIPH may serve as a prognostic biomarker for lung-cancer patients.

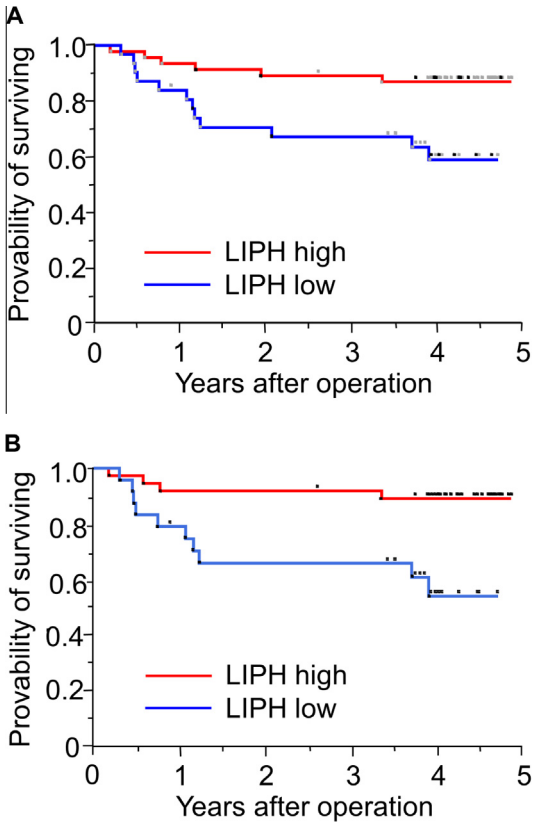

Fig. 4. LIPH constitutes a prognostic marker in lung cancer. Overall survival after surgery. (A) Survival curves of all 78 patients. (B) Survival curves of the 62 patients who did not receive adjuvant chemotherapy. LIPH high: patients with >6.0 ng/mL of LIPH protein in sera; LIPH low: patients with <6.0 ng/mL of LIPH protein in sera. Characteristics of cancer patients used for the analysis were shown in Table 2.

4. Discussion

The *LIPH* gene is located within 3q.27 region of chromosome 3 long arm which is reported as one of the most frequent areas of gene amplification in NSCLC [6]. In SCC, some of the tumor driver genes such as those encoding p63, phosphatidylinositol 3-kinase C catalytic subunit isoform-α (PI3KCA), and sex-determining region Y-box 2 (Sox2) are located at 3q [10–12]. The frequency of 3q.27 amplification in SCC has been reported to be around 30% [13,14]. In this study, we detected similar frequency of the up-regulation of *LIPH* gene expression by qPCR, suggesting that our data may also support the above findings. On the other hand, copy-number alterations are less frequent in AC patients [15]. However, our biochemical analysis and previous microarray data clearly indicated that LIPH was upregulated in many ACs and BACs. Frequent up-regulation of LIPH in lung AC may be due to the activation of gene transcription.

LIPH is a member of the mammalian triglyceride lipase family. LIPH specifically hydrolyzes phosphatidic acid producing 2-acyl lysophosphatidic acid (LPA) [16–18] which has growth factor-like biological activities [19,20]. Another LPA-producing enzyme, ATX, has been reported to be expressed in prostate and breast cancers, and in melanoma [18]. The receptor-mediated ATX-LPA signaling is associated with tumor initiation and progression [21]. In ATX-activated tumors, ATX-LPA signaling promotes cell proliferation and migration by activating the mitogen-activated protein kinase (MAPK) or PI3K/Akt pathways [22,23]. In addition, the LPA-mediated signaling in colorectal cancer cells was shown to induce transactivation of the epidermal growth factor receptor (EGFR) [24], which is another key event in lung cancer initiation and progression. However, in AC cell lines LIPH over-expression or knock down was not followed by any distinct changes in the activation of the EGF pathway components, such as phosphorylation of MAPK, Akt, or EGFR (data not shown). Although endogenous LIPH contributes to the proliferation of ACs, overexpression of LIPH gene failed to show significant promotion of the cell proliferation in several AC cell lines (Fig. 3). Thus, our results suggested that LIPH does not strongly promote the cellular proliferation of NSCLCs.

Immunohistochemical analysis demonstrated that LIPH was very frequently detected in NSCLC cells, like lung AC and BAC. This was much more frequent than those detected by qPCR. Because qPCR analysis with the tissue samples containing limited percentage of *LIPH*-positive cells has difficulty in showing significant up-regulation of the gene expression, immunohistochemical analysis could be more sensitive assay than qPCR analysis for the detection of LIPH-positive cells. Although the expression level was very low, LIPH could be detected in normal pulmonary alveolar cells such as type II alveolar epithelial cells and Clara cells (Fig. 2F). Interestingly, LIPH expression patterns in normal lungs as well as in tumor tissues shows some similarity to those of surfactant protein A (SP-A), a protein component of pulmonary surfactant [25]. SP-A has been widely used for immunohistochemical detection of lung AC [26,27]. Although the relevance of LIPH and SP-A expression patterns for lung cancer is not clear, they may possibly cooperate in the formation of AC.

Recent genome-wide siRNA-based screening using the NSCLC cell line A549 identified LIPC, a lipase related to LIPH. LIPC is specifically expressed in the liver, but not in the lungs, of healthy individuals. However, in NSCLC, LIPC can be occasionally detected

by immunohistochemistry. Interestingly, LIPC^{high} patients showed significantly slower disease progression than LIPC^{low} patients [28]. In this study, we observed similar positive correlation between serum LIPH level and improved survival after surgery. Although we do not know the reason for the high expression of these lipases in lung cancers with favorable prognosis, these proteins could serve as prognostic biomarkers for post-operative lung cancer patients.

In conclusion, LIPH expression is frequently upregulated in the sera and tissues of cancer patients with NSCLC, especially with AC and BAC. Moreover, high serum levels of LIPH were correlated with better survival in early phase lung-cancer patients after surgery. Thus, LIPH may constitute a prognostic biomarker of lung cancer.

Acknowledgments

Human lung cancer cell lines, LC-2/Ad, RERF-LC-KJ, RERF-LC-AI, EBC-1, LK-2, and Sq-1 cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. ABC-1, HARA, PC-3, RERF-LC-Ad1, RERF-LC-Ad2, RERF-LC-MS, RERF-LC-Sq1, and VMRC-LCD cells were also provided from JCRB Cell Bank.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.106.

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