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学　位　論　文

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（Delta-like 1 homolog（DLK1）の治療標的としての可能性と ^{125}I 標識抗 DLK1 抗体を用いた肺癌モデルにおける放射免疫療法への応用）

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Delta-like 1 homolog (DLK1) as a possible therapeutic target and its application to radioimmunotherapy using ^{125}I-labelled anti-DLK1 antibody in lung cancer models (HOT1801 and FIGHT004)

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The authors declare that they have no conflict of interest.

Abstract

Objectives:

Delta-like 1 homolog (DLK1) is a non-canonical Notch ligand known to be expressed in several cancers but whose role in lung cancer is not yet fully understood. We sought to confirm DLK1 expression in small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), and to examine DLK1's clinical significance. Furthermore, we examined the possible utility of DLK1 as a novel target in radioimmunotherapy (RIT).

Methods:

We retrospectively assessed the correlation between clinical features and DLK1 expression by immunohistochemistry in resected specimens from 112 patients with SCLC and 101 patients with NSCLC. Moreover, we performed cell and animal experiments, and examined the possibility of RIT targeting DLK1 in SCLC using iodine-125 (^{125}I) -labeled anti-DLK1 antibody, knowing that ^{125}I can be replaced with the alpha-particle-emitter astatine-211 (^{211}At).

Results:

In SCLC and NSCLC, 20.5% (23/112) and 16.8% (17/101) of patients (respectively) had DLK1-positive tumors. In NSCLC, DLK1 expression was associated with recurrence-free survival ($P<0.01$) but not with overall survival. In SCLC, there was no association between DLK1 expression and survival. In addition, ^{125}I-labeled anti-DLK1 antibody specifically targeted DLK1 on human SCLC tumor cell lines. Furthermore, ^{125}I-labeled anti-DLK1 antibody was incorporated into tumor tissue in a mouse model.

Conclusion:

A proportion of SCLC and NSCLC exhibits DLK1 expression. As a clinical feature, DLK1 expression could be a promising prognostic factor for recurrence in patients with resected NSCLC. In addition, DLK1 could serve as a new therapeutic target, including RIT, as suggested by our pilot study using a radiolabeled anti-DLK1 antibody in SCLC.

Keywords: lung cancer, DLK1, Notch ligand, radioimmunotherapy, astatine

1. Introduction

Lung cancer is the most common cancer in the world today and is a leading cause of death due to cancer [1]. Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. In developed countries, close to 70% of patients with NSCLC present with locally advanced or metastatic disease at the time of diagnosis, when curative treatment typically is no longer feasible [2]. Recently, new antineoplastic agents have been developed for the treatment of NSCLC by molecularly targeted therapies or immune checkpoint inhibitors (ICIs). However, following these treatments, most patients relapse with tumors that have acquired resistance, as is seen following cytotoxic chemotherapy. On the other hand, small-cell lung cancer (SCLC) is characterized by aggressive growth and poor prognosis. Treatment and survival of patients with SCLC has not changed substantially in more than 40 years. Although some of the patients with limited-disease (LD) SCLC have relatively good surgical outcomes [3], most patients are diagnosed with late-stage disease, at which point patient outcomes are quite poor. SCLC is rarely cured with surgical therapy alone, and systemic chemotherapy and radiotherapy remain a cornerstone of treatment [4]. However, SCLC rarely harbors genetic mutations rendering the tumors drug sensitive, in contrast to the case with other carcinomas, and the therapeutic effect on SCLC of chemotherapy remains limited [5]. The indications for ICIs are expanding, and this modality is expected to gain use as a new treatment for SCLC [6, 7]. However, ICIs have many problems, including the need for relevant biomarkers and the occurrence of adverse events. Therefore, development of new treatments is desperately needed.

In SCLC, Notch signaling has been recognized as a pathway involved in tumor growth, in addition to previously identified genetic alterations such as those in *TP53* and *RB1* [8]. Notch signaling is associated with stem cell differentiation, cell proliferation, and angiogenesis [9]. Notch signaling consists of the interaction between Notch receptors

and ligands, components of the Notch receptor/ligand family. Mammals have four types of Notch receptors (Notch 1-4) and five types of Notch ligands (DLL1, 3, and 4, and Jag1 and 2); these gene products are associated with oncogenic or tumor suppressive phenotypes because the role of Notch is tissue- and context-dependent [10]. In addition, expression of the Notch1 receptor in SCLC serves as a prognostic factor and is clinically important [11].

Currently, this Notch receptor/ligand family is expected to be a therapeutic target in SCLC. Delta-like ligand 3 (DLL3), one of Notch ligands, is rarely expressed in non-malignant adult tissues, but frequently is expressed in SCLC. DLL3 has been implicated in tumor proliferation [12]. Rubin et al. [12] and Morgensztern et al. [13] reported promising results in Phase 1 and Phase 2 studies of rovalpituzumab, a conjugate drug that combines an anti-DLL3 antibody with a proteolytic enzyme. In a pioneering study, anti-DLL3 antibody complexes (Rovalpituzumab-Tesirin) showed antitumor effects against SCLC. However, in the Phase 2 portion of that trial, 91% of Rova-T-treated patients experienced drug-related adverse events [13] and the Phase 3 portion of the trial unfortunately was terminated due to insufficient survival benefit.

The present study focused on lung cancer expression of Delta-like 1 homolog (DLK1), a non-canonical Notch ligand. In humans, DLK1 expression is thought to be lost in normal tissues during development from fetal cells to adult tissues [14, 15]. A previous small study reported that DLK1 is expressed in 21 out of 30 SCLC tumors [16].

We additionally evaluated DLK1 as a possible target of radioimmunotherapy (RIT). In recent years, RIT, which adds a cytotoxic effect by conjugating a radionuclide to an anti-tumor-antigen antibody, has attracted increasing attention. In hematology, yttrium-90 (^{90}Y) -labeled ibritumomab tiuxetan (Zevalin®) (a complex of rituximab, an antibody against non-Hodgkin lymphoma, and the beta-particle-emitting isotope ^{90}Y) has been employed clinically [17]. Subsequent literature has proposed the use of

alpha-emitting nuclides in RIT, since such reagents would provide more localized activity and higher tumor cytotoxicity [18, 19]. In addition, Oriuchi et al. [20] assessed the potential of RIT for acute myelogenous leukemia with ^{211}At, another alpha-emitting nuclide, by examining specific binding of labeled antibodies to tumor cells; we wondered if the indication for RIT with ^{211}At could be expanded to include lung cancer.

Therefore, using immunohistochemistry (IHC) on surgically resected specimens, we confirmed DLK1 expression first in SCLC, and subsequently in NSCLC, and examined the relationship between DLK1 expression and clinical features. Additionally, we assessed the possible utility of radiolabeled anti-DLK1 antibody conjugated with ^{211}At; such an antibody is expected to be bound to and taken up by DLK1-displaying tumors. Specifically, we used a ^{125}I-labeled anti-DLK1 antibody as a surrogate for RIT, given that iodine has chemical properties similar to those of astatine. This work represents the first report (to our knowledge) showing the possible utility in lung cancer of RIT targeting DLK1.

2. Materials and Methods

2.1. Tumor samples (Appendices Figure 1)

For SCLC characterization, we enrolled 112 patients who underwent surgery between February 2003 and December 2012 at the Fukushima Medical University Hospital and related hospitals. This study represented cooperative research between the Hokkaido Lung Cancer Clinical Study Group Trial (HOT) and the Fukushima Investigative Group for Healing Thoracic Malignancy (FIGHT). For NSCLC characterization, we enrolled 216 patients who underwent complete resection between January 2012 and December 2017 at the Fukushima Medical University Hospital. Among these 216 patients with NSCLC, the specimens from 121 cases could not be examined by hybridization with complementary DNA due to storage conditions or insufficient sample volume; these cases were excluded. To the remaining 95 cases with NSCLC, we added 6 cases that presented with large-cell neuroendocrine carcinoma (LCNEC), a tumor that derives from the same neuroendocrine cell-type as SCLC. Thus, a total of 101 NSCLC + LCNEC cases were included in the NSCLC group analysis.

2.2. DLK1 IHC and clinical characteristics

Paraffin-embedded tumor specimens were sectioned at 3-μm thicknesses using a microtome; the resulting sections then were subjected to immunoperoxidase staining. The sections were incubated overnight at 4 °C with a primary monoclonal anti-DLK1 antibody (clone DI-2-20, mouse IgG1; Lot 130613; Chiome Bioscience, Inc., Tokyo, Japan). The primary antibody then was detected using biotinylated secondary anti-mouse IgG antibody (BA-2000; Vector Laboratories, Burlingame, CA, USA) by the avidin-biotin-peroxidase complex method. The sections were washed several times in phosphate-buffered saline (PBS) after each step and counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan), dehydrated by passage

through a graded series of alcohol solutions, and mounted on glass slides. For each specimen, tumors with stained cell membranes were counted in micrographs using NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan).

Based on this IHC analysis, we defined DLK1-positive specimens as those in which 10% or more of the tumor exhibited complete, intense circumferential membrane staining, or those in which 10% or more of the tumor exhibited incomplete and/or weak/moderate circumferential membrane staining for DLK1. We defined DLK1-negative specimens as those in which less than 10% of the tumor exhibited incomplete faint/barely perceptible membrane staining, or no staining, for DLK1 [16, 21] (Figure 1). In addition, we retrospectively analyzed the clinical characteristics of the groups that were positive and negative for DLK1 expression. The data collected included the following parameters: age at surgery, sex, smoking history, lymph node metastasis, date of surgery, date of recurrence, last confirmed survival date, and date of death. Recurrence-free survival (RFS) was defined as the time from surgery to the first recurrence or death. Overall survival (OS) was defined as the time from surgery to death.

2.3. *DLK1* gene expression analysis

IHC permitted the classification of 95 NSCLC cases in which DLK1 expression could be assigned as positive or negative. We confirmed the relative quantity of *DLK1* mRNA in each of these cases by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). At the time of collection, a small segment (7 mm x 7 mm) of each surgical specimen was excised and frozen in liquid nitrogen. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) and a PureLink RNA Mini Kit (Thermo Fisher Scientific, Inc.) according to the respective manufacturer's instructions. RNA quantity was assessed using a Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.); samples with 260 nm/280 nm absorbance ratios of 1.8 or larger were

considered eligible for RT-PCR determination of relative mRNA expression. Two-step RT-qPCR was performed using a TaqMan RNA-to-CT 2-Step kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. To detect *DLK1* mRNA, a TaqMan gene expression assay (Hs00171584_m1; Thermo Fisher Scientific, Inc.) was used. The glyceraldehyde-3-phosphate dehydrogenase-encoding transcript (*GAPDH*, Hs03929097; Thermo Fisher Scientific, Inc.) was used as an endogenous control. Forty cycles of amplification were performed for each sample. The ΔΔCq method was applied for quantitative evaluation [22]. Cycle quantification (Cq) values were calculated using Step One Plus software (ver. 2.3; Thermo Fisher Scientific, Inc.). ΔCq was defined as the difference between *DLK1* Cq and *GAPDH* Cq, and ΔΔCq was defined as the ratio to the endogenous control sample. Signals undetected after 40 cycles were considered to have an expression of zero. The data were processed using Microsoft Excel software 2013 (Microsoft, Redmond, WA, USA). The ΔΔCqs were converted into log10 values as relative quantities (designated log ratios).

2.4. DLK1 expression in human cell lines

We confirmed DLK1 expression in the human SCLC cell line Lu-135 (JCRB0170; JCRB cell bank, Osaka, Japan) and the human neuroblastoma cell line SK-N-F1 (ATCC CRL-2142; ATCC, Tokyo, Japan) by IHC using the anti-DLK1 antibody as described above.

2.5. Humanized anti-DLK1 antibody and ^{125}I-conjugate binding to human tumor cells

Anti-DLK1 antibody (HuBA-1-3D, Lot 150203; Chiome Bioscience) and ^{125}I-labeled control antibody (Synagis, Lot 160331; Chiome Bioscience were labelled with ^{125}I by the chloramine-T method [23]; ^{125}I-labeled antibodies were purified using Disposable PD-10 Columns (GE Healthcare), yielding reagents with radiochemical purities exceeding 95%.

Cells of the Lu-135 and SK-N-F1 lines were distributed (separately) at $1x10^6$ cells/well in Deep Well plates. ^{125}I-labeled anti-DLK1 antibody or ^{125}I-labeled control antibody were added to each well. All reactions were performed in quadruplicate. In a separate well, we added either cell line in combination with a mixture of ^{125}I-labeled anti-DLK1 antibody and a 20-fold higher amount of unlabeled anti-DLK1 antibody. The plates were incubated at 37 °C for 3 hours. The cells then were washed with PBS without Magnesium and Calcium. Radioactivity incorporated into Lu-135 or SK-N-F1 cells then was measured. The radioactivity was expressed as % injected dose (ID) divided by the cell number (the $1x10^6$ cells used as the inoculum in each well), yielding a value representing the percent ID per cell after administration of ^{125}I-labeled anti-DLK1 antibody. Radioactivity in each cell line was measured with an automated-well gamma counter (2480 WIZARD, PerkinElmer Inc., Waltham, MA, USA).

2.6. Incorporation into tumor tissue and pharmacokinetics of the ^{125}I-labeled anti-DLK1 antibody in tumor-bearing model mice implanted with SK-N-F1 cells

We implanted male nude mice subcutaneously with SK-N-F1 ($1x10^7$ cells/animal) to create a tumor-bearing mouse model. On day 34 after implantation, we injected ^{125}I-labeled anti-DLK1 antibody into the tail vein of these tumor-bearing mice. Sub-groups of 4 mice each were euthanized (under anesthesia) at 1, 6, 24, and 48 hours after antibody injection and tissues were collected for pharmacokinetic analysis of the antibody. At the time of sacrifice, whole blood was collected from the heart using Heparin Sodium Injection (5,000 units/5 mL; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) as the anticoagulant, and a portion of the heparinized whole blood was used to isolate plasma. Tumors and organs or tissues (muscle, heart, lung, spleen, pancreas, white fat, testis, stomach, small intestine, colon, kidney, adrenal, liver, brown fat, submandibular gland, bone (femur), and brain) were recovered and weighed at necropsy.

Radioactivity was measured with an automated-well gamma counter. The radioactivity of the organs, tissues, fluid (whole blood or plasma), and tumor are presented as percent of administered radioactivity dose per gram (% ID / g). The organ, tissue, and tumor values also were normalized to the blood values (i.e., as organ:blood, tissue:blood, and tumor:blood ratios).

2.7. Statistical analyses

For the patient characteristics of individuals with SCLC and NSCLC, groups of two or three categorical variables were compared by the chi-squared test or by Fisher's exact test, respectively. The Mann–Whitney U test was used to compare the two groups for data generated by RNA-Seq, given the presence of outliers within each group. For survival analyses, Kaplan–Meier curves were compared using the Mantel–Cox log-rank test and hazard ratios were calculated using the Mantel–Haenszel method. In multivariate analysis, Cox proportional hazards regression modeling was performed. The Youden index was used to find the appropriate cut-off value from the receiver operating characteristic (ROC) curve. Radioactivity data are presented as mean ± SD. In comparing the experimental radioactivity data with those obtained in controls, a non-paired Student's t-test was used. P values <0.05 were considered statistically significant. All statistical analyses were performed as two-tailed tests using SPSS (ver. 21.0; IBM Corp., Armonk, NY, USA) or Prism (ver. 7; GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. DLK1 is expressed in a subset of SCLC and NSCLC tumors

The SCLC group (N=112) exhibited a median age of 70 years (range, 52 to 85 years); 87 (78%) of these patients were men. Ninety-six (91%) of the SCLC group were current or former smokers (Table 1). IHC showed that tumors from 23 patients (21%) were DLK1 positive (Figure 1A, B). DLK1 expression was nominally (P=0.07) but not significantly higher in patients with pure SCLC than in those with tumors in which SCLC was combined with another histological type. In addition, there was no significant difference in age, sex, smoking history, pathological stage, and so on between the DLK1-positive group and the DLK1-negative group.

The NSCLC patients (N=101) exhibited a median age of 70 years (range, 39 to 87 years); 60 (59%) of these patients were men. Sixty-four (63%) of the NSCLC group were current or former smokers (Table 2). IHC showed that tumors from 17 patients (17%) were DLK1 positive (Figure 1C-F). DLK1 expression was common in cases with lymph node metastasis (P=0.04), but there was no correlation with pathologic staging. In addition, DLK1 expression was nominally (P=0.07) more common in patients younger than 70 years, but this difference did not achieve significance.

3.2. Comparison of DLK1 expression by IHC and relative quantity of mRNA

The expression level of *DLK1* mRNA in one of 95 NSCLC cases was used as a reference. We then evaluated the level of mRNA expression in other cases relative to the level in that reference case. Median (interquartile range) of the normalized level of *DLK1* mRNA was 3.79 (0.56-757.71) in the DLK1-positive group, which was significantly higher than 0.52 (0.02-3.46) in the DLK1-negative group (P=0.02) (Figure 1G).

3.3. The expression of DLK1 correlates with poor RFS in NSCLC

In the SCLC group, there was no association between DLK1 expression (by IHC) and OS at any p-stage (IA–IV, P=0.88, Appendices Figure 2). In 69 patients with radical resection of p-stage I, there was no association between DLK1 expression and RFS or OS (Appendices Figure 3).

In the NSCLC group, DLK1 expression was associated with RFS (P<0.01) but not with OS at p-stage IA–IIIA (Figure 2). For prognostic factors such as age, gender, smoking history, p-stage, lymph node metastasis, histology, surgical procedure (lobectomy or sub-lobectomy), and DLK1 expression, multivariate analysis showed that DLK1-positive status (P<0.01), sub-lobectomy (P<0.01), and p-stage (P<0.01) were independent factors in poor RFS (data not shown). In addition, comparing RFS and OS for each p-stage, DLK1 expression was associated with poor RFS even in p-stage I alone (P=0.03), but there was no association with OS (Appendices Figure 4).

Furthermore the ROC curve was used to confirm the appropriate cut-off value, which indicates the relationship between DLK1 expression and postoperative recurrence. This showed that the appropriate cut-off value for DLK1 expression was 7.5% (Appendices Figure 5). However, due to the heterogeneity of IHC, it was difficult to set the cut off value at 7.5%. Therefore, we created survival curves with DLK1 expression cut off values of 1% and 5%, and compared them with the survival curve of 10% DLK1 expression. This confirmed that 10% is still a more useful value to predict recurrence (Appendices Figure 6, 7).

3.4. ^{125}I-labeled anti-DLK1 antibody shows specific binding to tumor cell lines

DLK1 protein was observed in the cell membrane of Lu-135 (SCLC) and SK-N-F1 (neuroblastoma) cells, but staining was not observed in negative controls (Figure 3A-D). We next examined the uptake of ^{125}I-labeled anti-DLK1 antibody into Lu-135 and SK-N-F1. Radioactive label accumulated to a six-fold higher level in SK-N-F1 than in

Lu-135. Furthermore, competition of ^{125}I-labeled anti-DLK1 antibody with unlabeled anti-DLK1 antibody significantly suppressed the uptake of ^{125}I-labeled anti-DLK1 antibody into Lu-135 and SK-N-F1 (Figure 3E). This observation indicated specificity for the binding of ^{125}I-labeled anti-DLK1 antibody to DLK1 on these tumor cell membranes.

3.5. ^{125}I-labeled anti-DLK1 antibody is specifically taken up into tumor tissue in vivo but distributes primarily to blood

In tumor-bearing mice implanted with SK-N-F1 cells, uptake of ^{125}I-labeled anti-DLK1 antibody into tumor tissue was observed as increases in radioactivity, yielding %ID/g values of 3.54, 5.64, 3.99, and 2.81 at 1, 6, 24, and 48 hours (respectively) after injection. However, these values were lower than those in the blood (%ID/g values of 35.19, 25.04, 14.44, and 10.16 at the respective time points), plasma (%ID/g values of 65.85, 46.26, 26.78, and 19.07) and some organs and tissues (Figure 4). The ratios of mean radioactivity in organ:blood remained largely unchanged in some organs (heart, spleen, kidney, adrenal, and liver; Appendices Figure 8), but rose for the ratio in tumor:blood as time passed. Notably, the ratios of mean radioactivity in tumor:blood peaked at 48 hours.

4. Discussion

In this study, we investigated the expression and clinical features of DLK1 in 112 patients with SCLC. To our knowledge, no previous studies have examined the level of DLK1 expression in as many SCLC patients as were included in the present study. In addition, we investigated the expression of DLK1 in patients with NSCLC. Our results revealed that a subset (20.5% and 16.8%, respectively) of tumors from patients with SCLC or from those with NSCLC exhibited staining for DLK1. Given these results, we focused on DLK1 as a possible therapeutic target for RIT in both SCLC and NSCLC. We performed what we believe to be the first cell and animal studies on the potential of DLK1 as a therapeutic target for RIT on the assumption of using ^{211}At.

Notch protein is present on the metazoan cell membrane, where the protein acts as a receptor. Delta and Jagged are known to be Notch ligands. Members of the Notch ligand family have been shown to play important roles in cell differentiation and proliferation [24, 25]. In recent years, members of the Notch ligand family have attracted attention as possible therapeutic targets.

It has been reported that DLK1, another member of the Notch ligand family, is widely expressed during fetal development, but expression of DLK1 is downregulated after birth [15]. DLK1 participates in development and differentiation in various cell types, organs [26], and processes, including adipogenesis [27], osteogenesis [28], and neovascularization or angiogenesis [29]. Furthermore, abnormal expression of DLK1 has been reported in several human cancer types [16, 30-32], suggesting that DLK1 may play an important role in tumor progression and metastasis.

Few reports have addressed the role of the Notch receptor/ligand family or DLK1 in lung cancer, and the expression of DLK1 in lung carcinomas and the clinical significance of this protein in lung cancer remain unexplored [16, 30, 33]. In the present study, the frequency of DLK1-positive SCLC tumors was no higher than that reported in

previous studies [16], but a certain proportion of NSCLC and SCLC tumors were positive for DLK1 expression, as judged by IHC. The relatively low frequency of DLK1-positive SCLC tumors may reflect the strict criteria used in our evaluation by IHC. Our results suggested that DLK1 expression may be a novel predictor for metastasis and postoperative recurrence in NSCLC. Indeed, DLK1 expression in NSCLC has been reported to affect the proliferation of tumor cells [30]. In addition, DLK1 expression has been suggested as an important prognostic factor in ovarian cancer [34]. Our results support these previous inferences.

Furthermore, our results indicated the possible utility of DLK1 as a target for RIT employing nuclides that selectively damage lung cancer tumor cells. Although beta-particle therapy already has been put into practical use for the treatment of non-Hodgkin lymphoma [17], a therapeutic that only creates single-strand breaks in the DNA of tumor cells is unlikely to kill the tumor. Beta-particle therapy for solid cancers has not yet been established. On the other hand, alpha-particle therapy has high tumor toxicity because of this modality's extremely high radiation energy, which is capable of creating double-strand breaks in tumor cell DNA [35]. As an alpha-particle source, ^{223}Ra has already been put into practical use for treatment of bone metastases of prostate cancer [36], and ^{225}Ac has been shown to have strong antitumor effects against prostate cancer [19]. Furthermore, in recent years, ^{211}At has been considered to be one of the more attractive alpha-emitters in this regard, given this nuclide's half-life of 7.2 hours, which is long enough to permit incorporation into a monoclonal antibody while still providing high levels of alpha-particle emission during decay [37, 38]. In addition, ^{211}At has been also noted to have less serious toxicity compared with beta-particle therapy because of the shorter effective range of alpha-particles [39, 40].

The production of ^{211}At requires a special cyclotron, and there are few production bases around the world. However, our institute is one of the rare production sites for ^{211}At.

Thus, only a limited number of previous reports on RIT incorporating ^{211}At have been published. Nonetheless, ^{211}At has shown strong antitumor effects in animal models of disseminated ovarian cancer [18] and of neuroblastoma [41], and there are high expectations for the therapeutic application of this nuclide.

Since ^{211}At is in the same element group (halogens) as iodine, the same molecular design is possible as with iodine preparations. In the current study, we confirmed that an ^{125}I-anti-DLK1 antibody complex was successfully taken up into tumor cells, representing the first demonstration (to our knowledge) of the possible therapeutic application of ^{211}At targeting of DLK1 on tumor cells. Our observation in a mouse model of an increase over time in the tumor:blood radioactivity ratio suggested that ^{125}I-labeled anti-DLK1 antibody is specifically incorporated into tumor tissue. However, our results also revealed non-specific binding of the labeled antibody to other organs and tissues. In this study, antibody complexes showed specific binding to DLK1 on tumor cells in-vitro, and this observation could serve as the basis of development for RIT. However, concerns regarding the distribution and accumulation of antibody complexes in-vivo represent a challenge for the implementation of the proposed targeting of DLK1, and are a possible limitation of this study. This challenge will need to be addressed in future experiments.

5. Conclusions

DLK1 expression was observed in a subset of tumors from patients with SCLC or NSCLC. In NSCLC, the expression of DLK1 correlated with lymph node metastasis and poor RFS, suggesting that DLK1 expression may be a predictor for metastasis and postoperative recurrence. Furthermore, specific binding by antibody complexes targeting DLK1 was observed in SCLC, suggesting a possible new therapeutic target. In addition, the results represent the possible development of anti-DLK1 antibody for RIT with ^{211}At.

Compliance with ethical standards

Ethical approval and ethical standards: This study was approved by the Institutional Ethics Committee at Fukushima Medical University (Approval Nos. 29270 and 29080).

Informed consent: Patients with lung cancer provided written informed consent for the use of tissue specimens and clinical data for research prior to undergoing pulmonary resection at the Department of Chest Surgery of Fukushima Medical University.

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Table 1. Patient characteristics, including correlation between DLK1 expression and clinicopathological features of patients with SCLC

Factors	DLK1 positive N=23 (21%)	DLK1 negative N=89 (79%)	P-value
Age at surgery			
<70	9 (39%)	43 (48%)	
≥70	14 (61%)	46 (52%)	0.49
Sex			
Male	20 (87%)	67 (75%)	
Female	3 (13%)	22 (25%)	0.28
Smoking status			
Former or Current	21 (96%)	75 (90%)	
Never	1 (4%)	8 (10%)	0.68
p-stage (7th)			
I	15 (65%)	54 (61%)	
II	2 (9%)	14 (16%)	
III	5 (22%)	19 (21%)	
IV	1 (4%)	2 (2%)	0.71
Lymph node metastasis			
pN-	15 (65%)	56 (67%)	
pN+	8 (35%)	28 (33%)	1.00
Histology			
Pure SCLC	20 (87%)	60 (67%)	
Combined SCLC	3 (13%)	29 (33%)	0.07

Table 2. Patient characteristics, including correlation between DLK1 expression and clinicopathological features of patients with NSCLC

Factors	DLK1 positive N=17 (17%)	DLK1 negative N=84 (83%)	P-value
Age at surgery			
<70	12 (71%)	38 (45%)	
≥70	5 (29%)	46 (55%)	0.07
Sex			
Male	7 (41%)	53 (63%)	
Female	10 (59%)	31 (37%)	0.11
Smoking status			
Former or Current	8 (47%)	56 (67%)	
Never	9 (53%)	28 (33%)	0.17
p-stage (7th)			
I	10 (59%)	66 (79%)	
II	3 (18%)	8 (9%)	
III	4 (23%)	10 (12%)	0.23
Lymph node metastasis			
pN-	10 (59%)	70 (83%)	
pN+	7 (41%)	14 (17%)	0.04
Histology			
Adenocarcinoma	14 (82%)	56 (67%)	
Squamous cell carcinoma	2 (12%)	21 (25%)	
Others	1 (6%)	7 (8%)	0.56

Figure captions

Figure 1. IHC images and *DLK1* mRNA expression in patient tissues.

Representative IHC images for (A) DLK1-positive SCLC, (B) DLK1-negative SCLC, (C) DLK1-positive adenocarcinoma, (D) DLK1-negative adenocarcinoma, (E) DLK1-positive squamous cell carcinoma, and (F) DLK1-negative squamous cell carcinoma. (G) Relative *DLK1* mRNA expression as assessed by RT-PCR in tumors judged (by IHC) to belong to the DLK1-positive group (N=16) and the DLK1-negative group (N=79). Median (interquartile range) was 3.79 (0.56-757.71) in the DLK1-positive group and 0.52 (0.02-3.46) in the DLK1-negative group (P=0.02).

IHC: immunohistochemistry

RNA: ribonucleic acid

DLK1: Delta-like 1 homolog

RT-PCR: reverse transcription-quantitative polymerase chain reaction

Figure 2. Kaplan–Meier survival curves for all enrolled patients with NSCLC and LCNEC. (The cut-off value for DLK1 expression was 10%)

(A) Comparison of RFS between patients in whom tumors were positive (N=17) and negative (N=84) for DLK1 expression (P<0.01). (B) Comparison of OS between patients in whom tumors were positive (N=17) and negative (N=84) for DLK1 expression (P=0.12). (The cut-off value for DLK1 expression was 10%)

NSCLC: non-small-cell lung cancer

LCNEC: large-cell neuroendocrine carcinoma

RFS: recurrence-free survival

OS: overall survival

DLK1: Delta-like 1 homolog

Figure 3. IHC images and the uptake of ^{125}I-labeled anti-DLK1 antibody in cell-lines.

Representative IHC images for (A) Lu-135 (SCLC cell line), (B) SK-N-F1 (neuroblastoma cell line), and the respective negative controls (C, D). The IHC analysis employed the DI-2-20 anti-DLK1 antibody diluted 1:3000 (A) and 1:14000 (B). For the negative controls, the incubation step with the primary antibody was omitted (C for Lu135; D for SK-N-F1). (E) The uptake of ^{125}I-labeled anti-DLK1 antibody into Lu-135 and SK-N-F1 was measured; values of radioactivity (%ID/g) are expressed as mean ± SD. Competition of ^{125}I-labeled anti-DLK1 antibody with unlabeled anti-DLK1 antibody suppressed the uptake of ^{125}I-labeled anti-DLK1 antibody into Lu-135 and SK-N-F1 (**P<0.01).

DLK1: Delta-like 1 homolog

IHC: immunohistochemistry

SCLC: small-cell lung cancer

Figure 4. Biodistribution of ^{125}I-labeled anti-DLK1 antibody in SK-N-F1 tumor-bearing mice.

The pharmacokinetics of the antibody at 1, 6, 24, and 48 hours post-dose were assessed in vivo. Using a gamma counter, we measured the radioactivity (%ID/g) in fluids (whole blood, plasma), tumors, and organs or tissues (as indicated); values are expressed as mean ± SD of the radioactivity.

DLK1: Delta-like 1 homolog

Appendices Fig. 1 Enrollment in this study of patients with SCLC and NSCLC.

SCLC patient samples were collected at hospitals participating in the Hokkaido Lung Cancer Clinical Study Group Trial (HOT) or the Fukushima Investigative Group for Healing Thoracic Malignancy (FIGHT). NSCLC patient samples were collected at the

Fukushima Medical University Hospital.

SCLC: small-cell lung cancer

NSCLC: non-small-cell lung cancer

Appendices Fig. 2 Kaplan–Meier overall survival (OS) curves for all enrolled patients with SCLC.

Comparison of OS between patients in whom tumors were positive (N=23) or negative (N=89) for DLK1 expression (P=0.88).

SCLC: small-cell lung cancer

DLK1: Delta-like 1 homolog

Appendices Fig. 3 Kaplan–Meier survival curves for patients with SCLC in p-stage I.

(A) Comparison of RFS between patients in whom tumors were positive (N=15) or negative (N=53) for DLK1 expression (P=0.87). (B) Comparison of OS between patients in whom tumors were positive (N=15) or negative (N=53) for DLK1 expression (P=0.98).

SCLC: small-cell lung cancer

RFS: recurrence-free survival

OS: overall survival

DLK1: Delta-like 1 homolog

Appendices Fig. 4 Kaplan–Meier survival curves for patients with NSCLC in p-stage I.

(A) Comparison of RFS between patients in whom tumors were positive (N=10) or negative (N=66) for DLK1 expression (P=0.03). (B) Comparison of OS between patients in whom tumors were positive (N=10) or negative (N=66) for DLK1 expression (P=0.77).

NSCLC: non-small-cell lung cancer

RFS: recurrence-free survival

OS: overall survival

DLK1: Delta-like 1 homolog

Appendices Fig. 5 ROC curve for setting the appropriate DLK1 expression cut-off value.

The relationship between the expression rate of DLK1 by IHC and postoperative recurrence was evaluated using the ROC curve. The appropriate cut-off value for DLK1 expression was 7.5%.

ROC: receiver operating characteristic

DLK1: Delta-like 1 homolog

IHC: immunohistochemistry

Appendices Fig. 6 Kaplan–Meier survival curves for all enrolled patients with NSCLC and LCNEC. (The cut-off value for DLK1 expression was 1%)

(A) Comparison of RFS between patients with tumors positive for DLK1 expression of 1% or more (N=36) and patients with negative DLK1 expression of less than 1% (N=65) (P=0.50). (B) Comparison of OS between patients with tumors positive for DLK1 expression of 1% or more (N=36) and patients with negative DLK1 expression of less than 1% (N=65) (P=0.48).

NSCLC: non-small-cell lung cancer

LCNEC: large-cell neuroendocrine carcinoma

RFS: recurrence-free survival

OS: overall survival

DLK1: Delta-like 1 homolog

Appendices Fig. 6 Kaplan–Meier survival curves for all enrolled patients with NSCLC and LCNEC. (The cut-off value for DLK1 expression was 5%)

(A) Comparison of RFS between patients with tumors positive for DLK1 expression of 5% or more (N=27) and patients with negative DLK1 expression of less than 5% (N=74) (P=0.04). (B) Comparison of OS between patients with tumors positive for DLK1 expression of 5% or more (N=27) and patients with negative DLK1 expression of less than 5% (N=74) (P=0.11).

NSCLC: non-small-cell lung cancer

LCNEC: large-cell neuroendocrine carcinoma

RFS: recurrence-free survival

OS: overall survival

Appendices Fig. 8 The tumor:blood and organ:blood ratios of mean ^{125}I-labeled anti-DLK1 antibody radioactivity.

In heart, spleen, kidney, adrenal, and liver, the blood-normalized values (ratios) were largely unchanged over time, while the tumor:blood ratios increased over time, peaking at 48 hours.

DLK1: Delta-like 1 homolog

Figure 1.

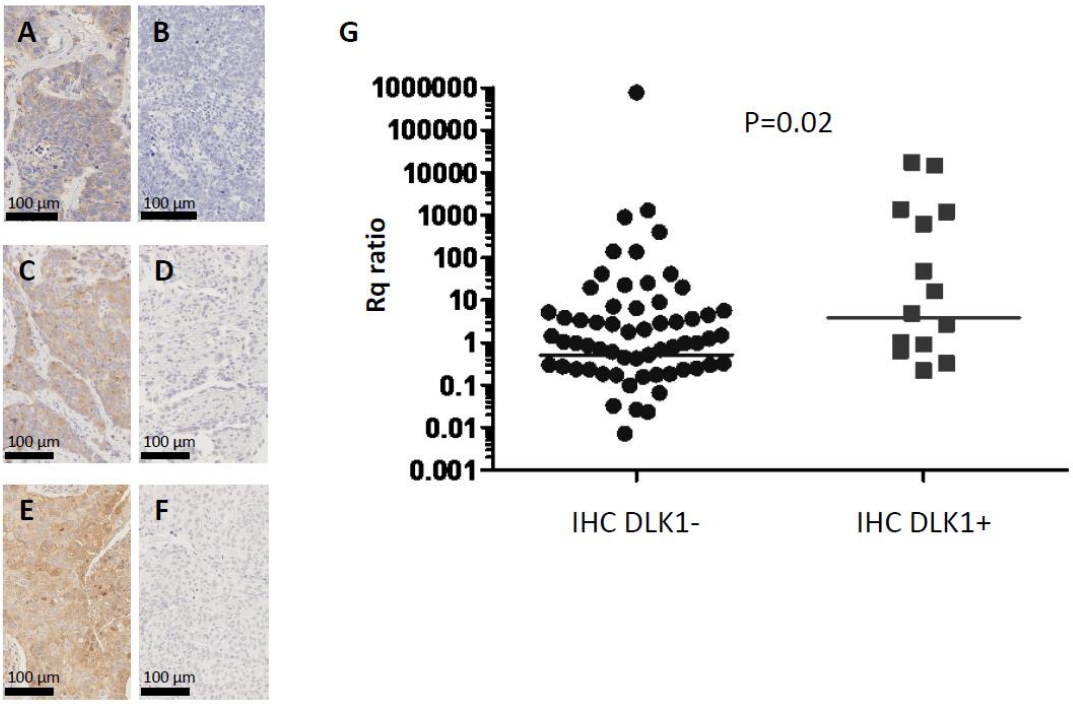


Figure 2.

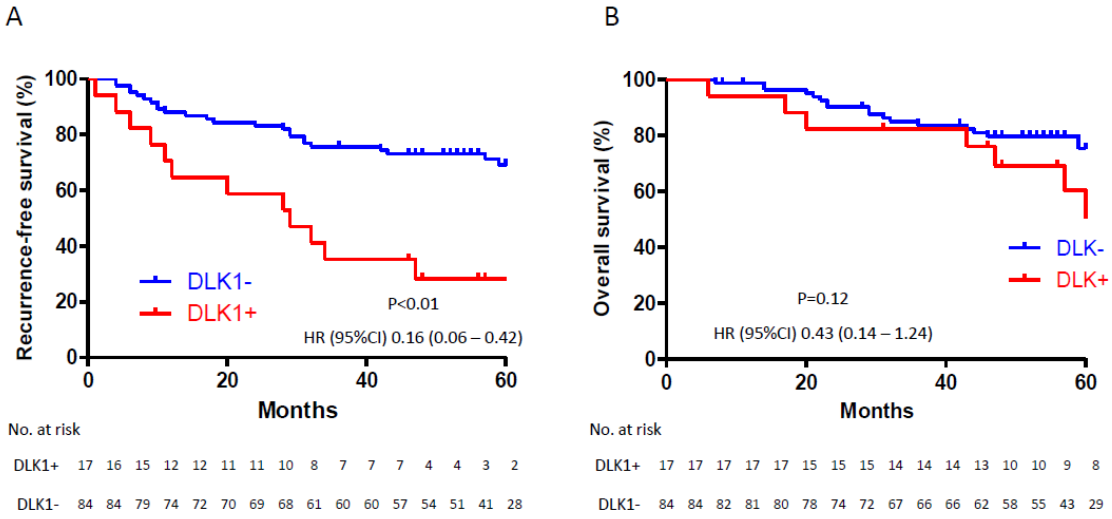


Figure 3.

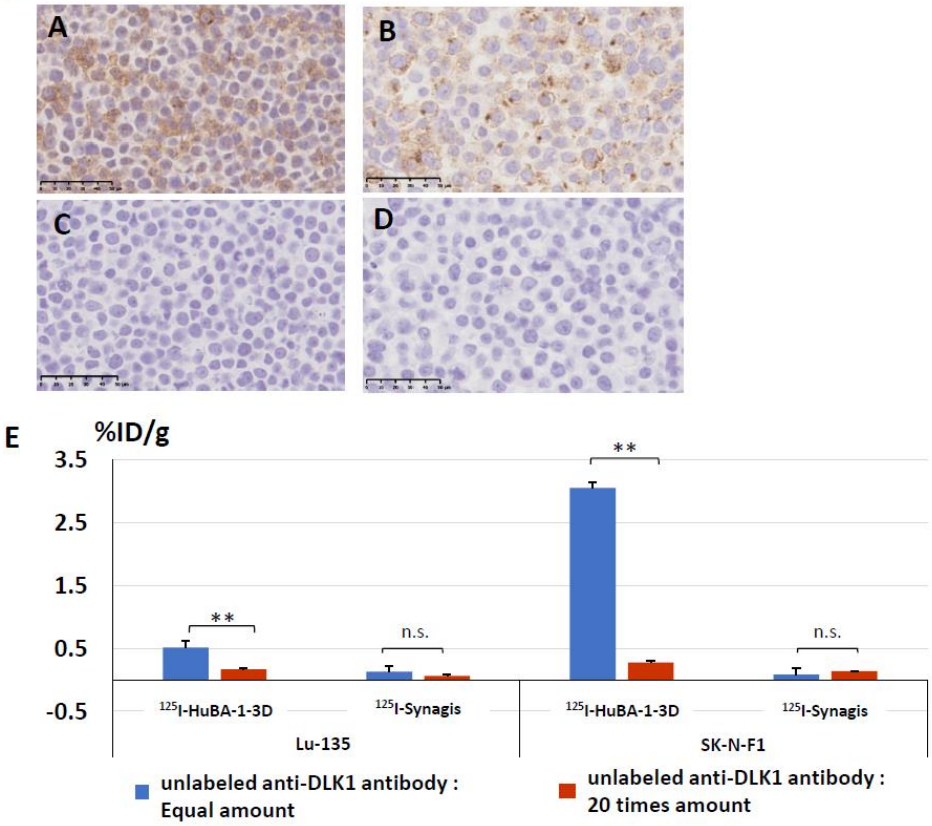
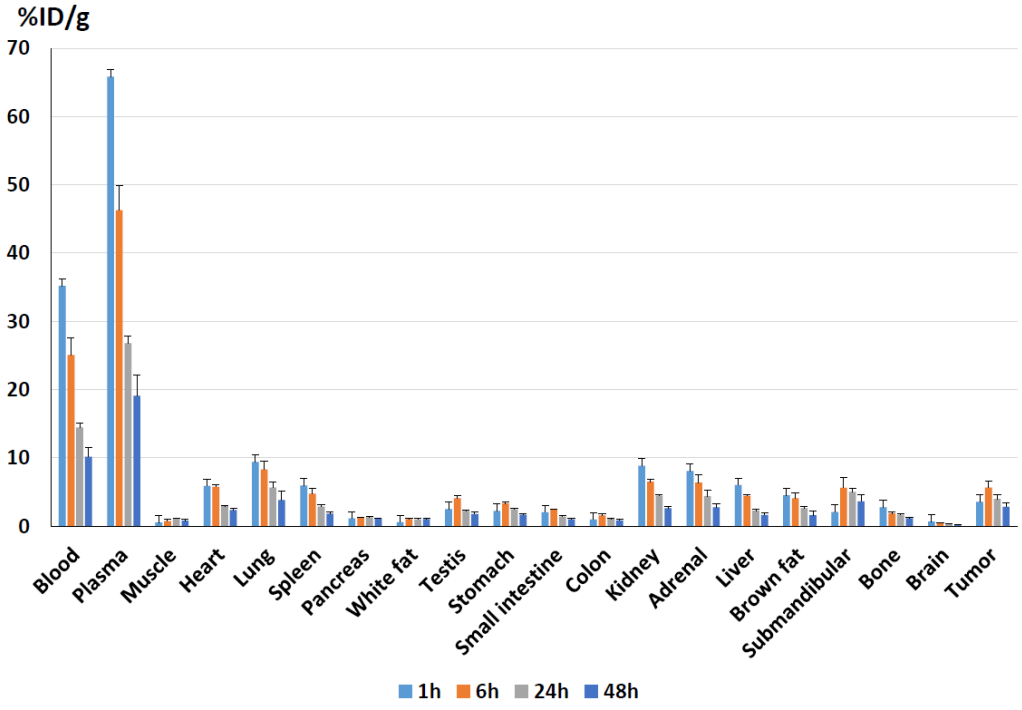
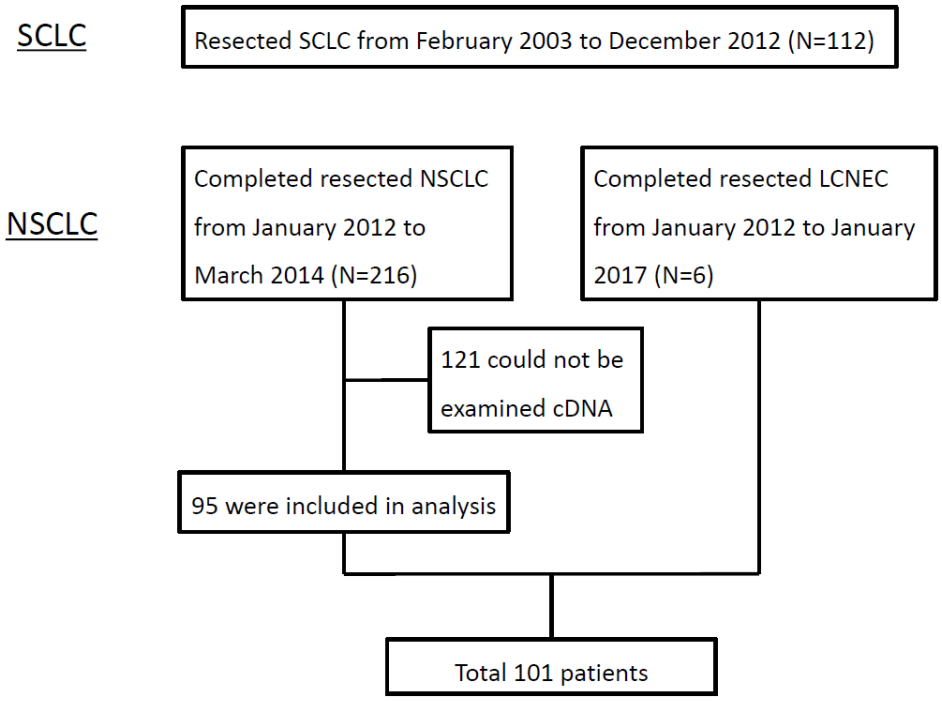


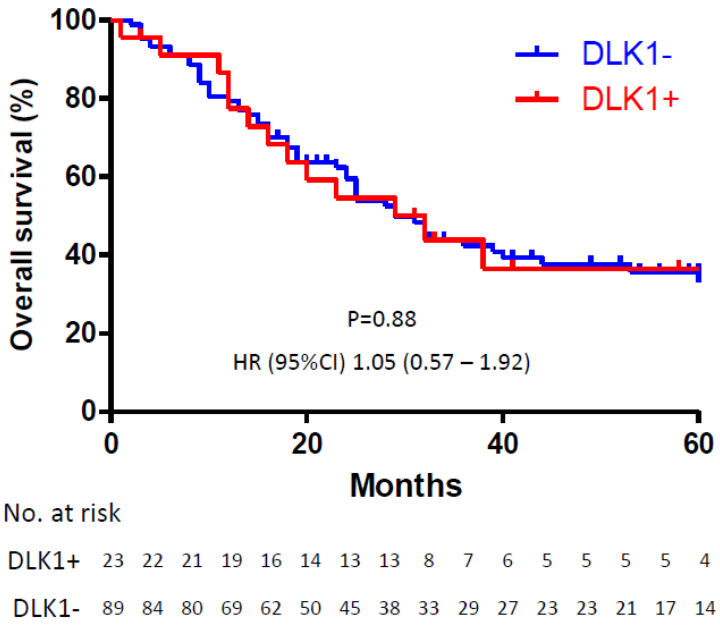
Figure 4.



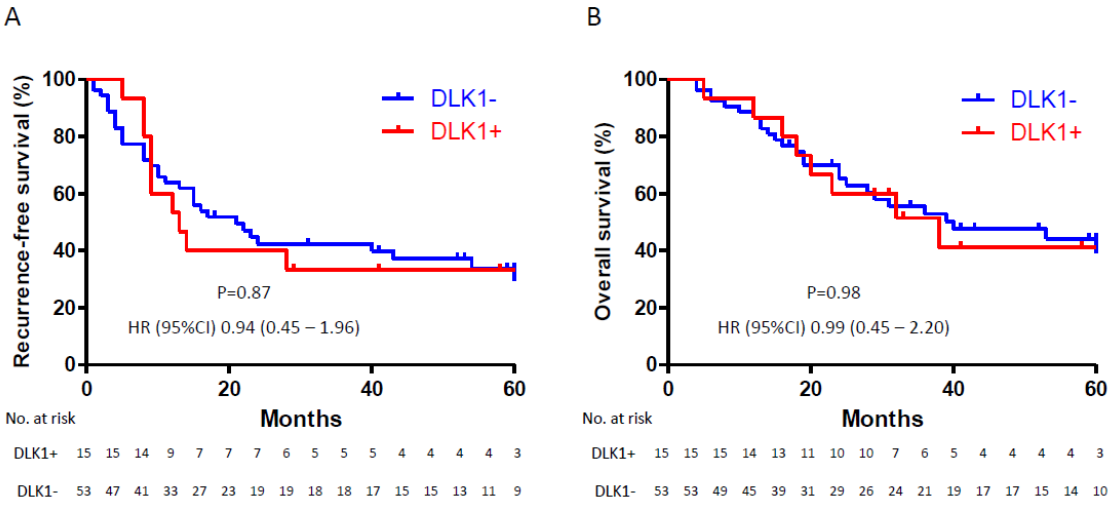
Appendices Figure 1.



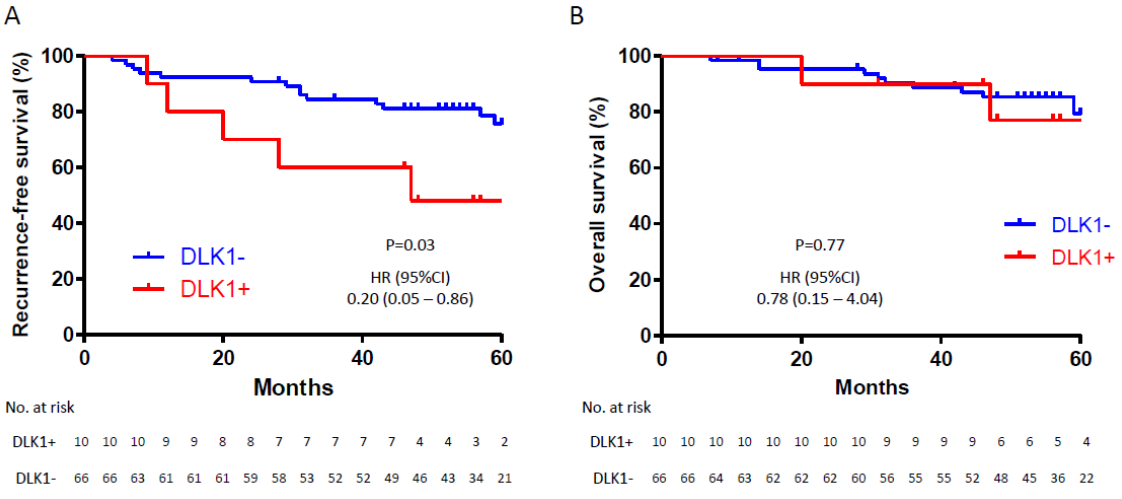
Appendices Figure 2.



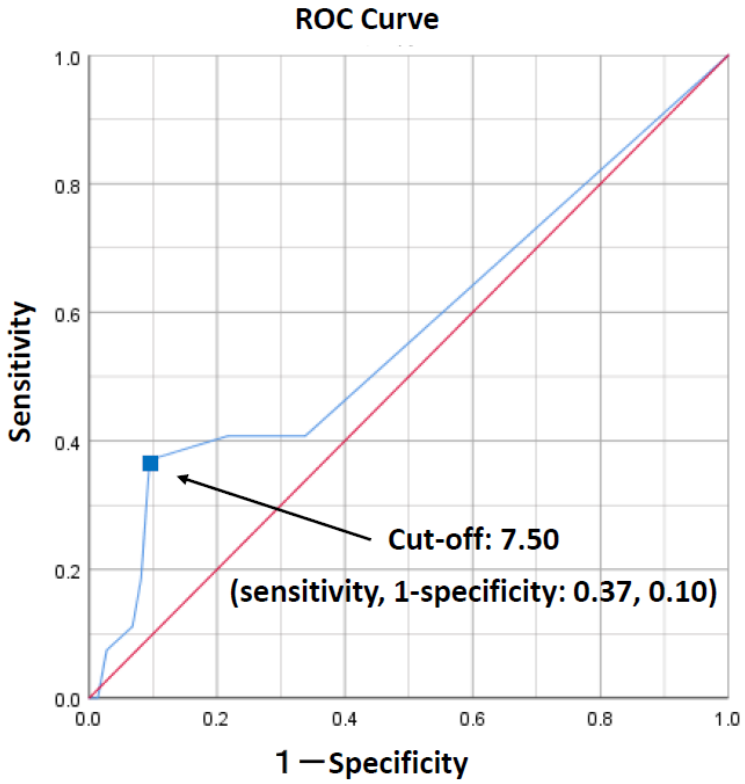
Appendices Figure 3.



Appendices Figure 4.



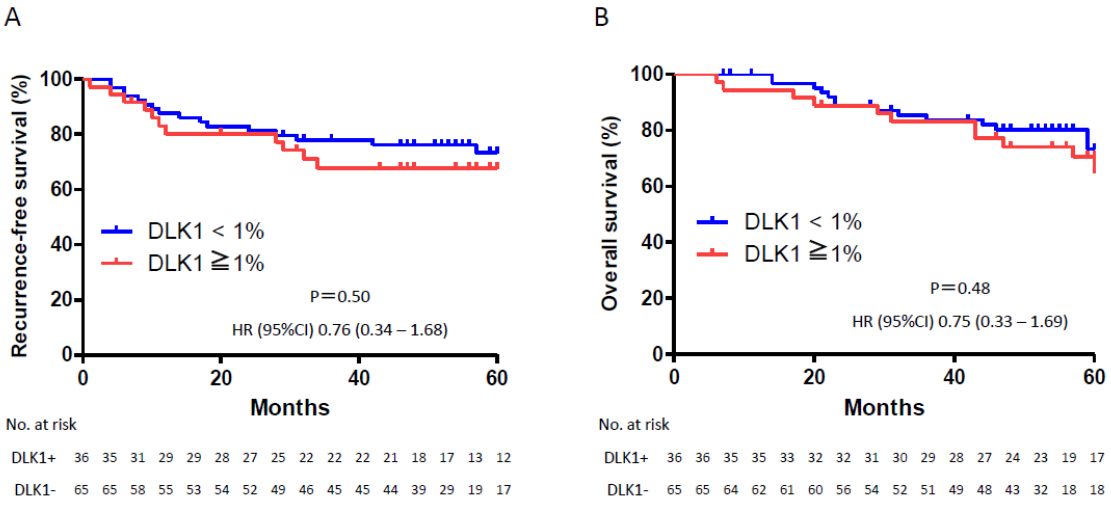
Appendices Figure 5.



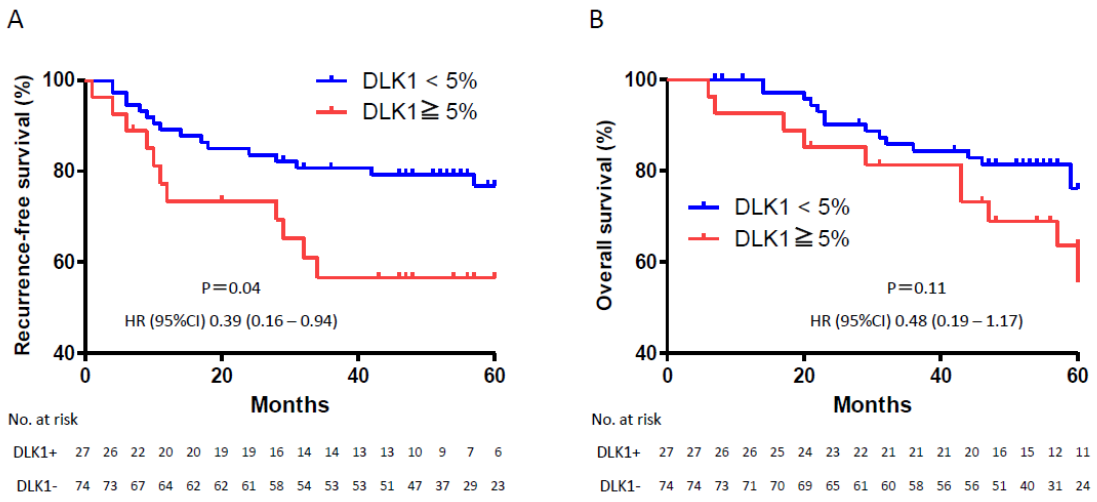
Area: 0.573

(95%CI: 0.44 – 0.71)

Appendices Figure 6.



Appendices Figure 7.



Appendices Figure 8.