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SIRT6 Expression is Associated With Poor Prognosis and Chemosensitivity in Patients With Non-Small Cell Lung Cancer

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Background: Despite advances in the development of various therapeutic agents, non-small cell lung cancer (NSCLC) is associated with a poor prognosis. To improve the prognosis of patients with NSCLC, new therapeutic targets for overcoming drug resistance are required. The process of autophagy is required to support the tumorigenesis and drug resistance of cancer cells. We investigated the clinical significance of SIRT6, a member of the NAD^+-dependent deacetylase family, which regulates a variety of cancer-related processes, including autophagy.
Methods: Immunohistochemistry analysis of SIRT6 expression and localization in 98 NSCLC clinical specimens and in vitro analysis using SIRT6-knockout lung carcinoma cell lines were performed.
Results: Patients with high cytoplasmic expression and low nuclear expression of SIRT6 ($n = 33$) had more aggressive cancer, shorter overall survival, and shorter recurrence-free survival than did patients with different SIRT6 expression profiles ($P < 0.05$). In vitro analysis revealed that SIRT6 knockdown lung adenocarcinoma cell line improved paclitaxel sensitivity ($P < 0.05$) and reduced the expression levels of both nuclear factor kappaB and autophagy marker Beclin1.
Conclusion: Our data demonstrated that SIRT6 expression in NSCLC could be a useful prognostic marker and that SIRT6 might represent a novel target gene for predicting sensitivity of chemotherapy in lung adenocarcinoma.
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KEY WORDS: **sirtuin; sirtuin 6; autophagy; chemoresistance**

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) represents approximately 85% of all cases of lung cancer [1]. Targeted therapies such as epidermal growth factor receptor tyrosine kinase inhibitors (EFGR-TKIs) have become standard therapeutic agents, improving clinical outcomes and therapeutic options for patients with NSCLC [2]. However, the prognosis for patients with advanced NSCLC, which does not respond to conventional chemotherapies or EGFR-TKIs, remains very poor. The discovery of suitable biomarkers for predicting prognosis and chemosensitivity may be important for monitoring cancer recurrence and providing information on appropriate adjuvant or neoadjuvant chemotherapies. Therefore, further research is needed to identify new therapeutic targets and tools for overcoming NSCLC with drug resistance.

The seven members of the sirtuin family (SIRT1-7) belong to the family of NAD^+-dependent deacetylases and are classified as class III histone deacetylases [3]. These enzymes are widely expressed in mammalian cells and modulate various biological processes, including cell survival, development, chromatin dynamics, DNA repair, metabolism, and cancer [4]. SIRT6 is predominantly localized in the nucleus of various cells and regulates transcription, genomic DNA stability and repair, metabolism, and aging through its histone deacetylation function [5]. Moreover, SIRT6 has been reported to regulate autophagy [6], an important process in cancer cell survival and chemoresistance, and has attracted attention for its function in overcoming drug resistance in hormone- and drug-refractory cancers [7]. SIRT6 has been shown to be downregulated in clinical samples from pancreatic cancer, colorectal cancer, head and neck squamous cell carcinoma, and hepatocellular carcinoma [8–10], and may function as a tumor suppressor. Other reports have shown that prostate cancer and breast cancer patients with high SIRT6 expression have significantly poor prognoses and that prostate and breast cancer cells expressing SIRT6 show resistance to anticancer drugs [11,12]. However, few studies have investigated the relationship between SIRT6 expression levels and clinical outcomes in NSCLC.

The purpose of this study was to clarify the clinical significance of SIRT6, including its potential role in mediating anticancer drug sensitivity, in NSCLC. Therefore, we performed immunohistochemistry analysis of 98 clinical NSCLC samples to evaluate the relationship between SIRT6 expression and clinicopathological features, prognosis, and survival times after anticancer drug treatment. Furthermore, we explored the in vitro effects of siRNA-mediated SIRT6 knockdown on paclitaxel sensitivity and autophagy in human NSCLC cell lines.

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MATERIALS AND METHODS

Clinical Samples and Cell Lines

We analyzed tumor specimens from 98 patients with lung cancer who underwent surgery for excision of a primary tumor between January 1999 and February 2006 in the Department of General Surgical Science of Gunma University School of Medicine. The patients included 65 men and 33 women with a median age of 65 years (range, 32–84 years) at surgery. Sixty-five patients were former/current smokers, with a median Brinkman index (BI; number of cigarettes per day times years) of 1,051, and 33 patients had no history of smoking. Eighty-one patients had adenocarcinomas, and 17 had squamous cell carcinomas. Fifty-seven patients had stage I lung cancer, 9 patients had stage II lung cancer, 29 patients had stage III lung cancer, and 3 patients had stage IV lung cancer at the time of surgery. Forty-eight patients received chemotherapy after operation, and eleven patients received radiation after operation. All patients provided written informed consent.

The human lung adenocarcinoma cell lines A549, H1975, and H2009 and the lung squamous cell carcinoma cell lines EBC-1 and RERF-LC-AI were all maintained in RPMI1640 medium containing 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX) and 100 U/ml penicillin and streptomycin sulfate (Life Technologies, Carlsbad, CA). Cells were cultured in a humidified incubator with 5% CO_2 at 37°C. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) or Riken BioResource Center (Japan). It was previously validated that these cells were not cross-contaminated with other cell lines by STR-PCR in each cell bank.

Immunohistochemistry

The resected surgical specimens were fixed with 10% formaldehyde, embedded in paraffin blocks, cut into 4-μm-thick sections, and mounted on glass slides. Staining was performed using standard methods, and detection was facilitated by formation of the streptavidin-biotin peroxidase complex (S-ABC). All sections were incubated at 60°C for 60 min, deparaffinized in xylene, rehydrated, and then incubated with fresh 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to block endogenous peroxidase activity. After rehydration through a graded ethanol series, antigen retrieval was carried out in 10 mM citrate buffer (pH 6.4) at 98–100°C for 20 min. The sections were then passively cooled to 30°C. After rinsing the sections in 0.1 M phosphate-buffered saline (PBS, pH 7.4), nonspecific binding sites were blocked by incubating the sections with 10% normal goat serum for 30 min. The sections were then incubated overnight at 4°C and at room temperature for 30 min with mouse monoclonal anti-SIRT6 antibodies (Abnova, Taiwan) at a dilution of 1:200 in PBS containing 1% bovine serum albumin (Sigma–Aldrich, St. Louis, MO). The sections were washed in PBS, incubated with biotinylated anti-mouse IgG, A, M solution (Nichirei Co., Tokyo, Japan) for 30 min at room temperature, and finally incubated in S-ABC solution (Nichirei Co.) for 30 min. The chromogen, 3,3′-diaminobenzidine tetrahydrochloride, was applied as a 0.02% solution containing 0.005% hydrogen peroxide in a 50 mM ammonium acetate-citrate acid buffer (pH 6.0). The sections were lightly counterstained in Mayer's hematoxylin and mounted on glass slides.

The level of SIRT6 immunoreactivity was defined as follows: (i) low expression = no staining, weak staining or strong complete cytoplasm/nuclear staining in <20% of tumor cells; (ii) high expression = strong complete cytoplasm/nuclear staining in ≥20% of tumor cells. The expression levels were evaluated by two independent investigators who reached a consensus for all samples.

Knockdown of SIRT6 Expression by RNA Interference

SIRT6-specific small interfering RNA (siRNA) #1 (5′-GGGAC-AAACUGGCAGAGC AGCUCUGCCAGUUUGUCC-3′), siRNA #2 (5′-GGAAGAAUGUGCCAAGUGACACUUGGCACAUUCUUC-3′), and negative control siRNA (siBonac Negative Control 1; scrambled siRNA) were purchased from Bonac and Hokkaido System Science (Japan), respectively. RNA interference assays for the target cell lines EBC-1 and A549 were performed using an in vitro electroporation protocol. In brief, the cells were suspended in Opti-MEM I (Life Technologies) without serum at a density of 1×10 cells/ml. siRNA was added to the cell suspension at a concentration of 1.5 μM. Next, 100 μl of the cell suspension was transferred to a 2-mm gap cuvette electrode and then subjected to electroporation using an electroporator (CUY21EDIT II; BEX Co., Ltd., Tokyo, Japan). The conditions for electroporation were as follows: one pulse of 125 V with a 10-ms duration and 40-ms interval; followed by five pulses of 10 V with a 50-ms duration and 50-ms interval at 940 μF capacity; and five pulses of 10 V with a 50-ms duration and 50-ms interval at 940 μF capacity with reversed polarity.

Proliferation and Drug Sensitivity Assay

Proliferation and drug sensitivity assays were performed using cells that had been transfected with siRNA targeting *SIRT6* transcripts. For proliferation assays, EBC-1 cells were plated at approximately 1,500 cells/well and A549 cells were plated at 3,000 cells/well in 96-well plates with 100 μl of medium. The water-soluble tetrazolium (WST)-8 assay (Dojindo Laboratories, Tokyo, Japan) was used to quantify cell viability. Ten microliters of cell-counting solution was added to each well, and the plates were incubated at 37°C for 2 hr. The cell proliferation rate was then determined by measuring the absorbance of the medium at 450 nm with the reference wavelength set at 650 nm. The absorbance values were read using a microtiter plate reader (Molecular Devices, Sunnyvale, CA). A549 and EBC-1 cells were plated at approximately 3,000 cells/well in 96-well plates with 100 μl of medium in each well. Various concentrations of paclitaxel (Sawai Seiyaku, Osaka, Japan) were added (EBC-1 cells: 0, 5, 10, 20, 40, or 60 nM; A549 cells: 0, 25, 50, 100, 200, or 400 nM). After incubation for 48 hr at 37°C, cell viability was quantified by WST-8 assay using the same method as described for the proliferation assay above. Each experiment was carried out with 10 replicate wells for each concentration and experiments were repeated twice.

Protein Extraction and Western Blot Analysis

Western blot analysis was used to confirm the expression of target proteins using anti-SIRT6 (Cell Signaling Technology, Danvers, MA), anti-NFκB (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Beclin1 (Novus Biologicals, Littleton, CO), anti-BCL2 (Cell Signaling Technology), and anti-β-actin (Sigma, St. Louis, MO) antibodies in each lung cancer cell line. Four hundred micrograms of total protein was extracted with PROPREP protein extraction solution (iNtRON Biotechnology, Inc., Korea). Total protein was then electrophoresed on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes using a wet transfer protocol. The membranes were blocked with 5% skim milk, and the proteins were detected using anti-SIRT6 rabbit monoclonal antibodies (1:1,000), anti-NFκB rabbit polyclonal antibodies (1:200), anti-Beclin1 rabbit polyclonal antibodies (1:1,000), and anti-BCL2 rabbit polyclonal antibodies (1:1,000). Anti-β-actin mouse monoclonal antibodies (clone AC-74; 1:2,000) served as a control. Bands were detected, and band intensities were calculated using ECL Prime Western blotting detection reagent (GE Healthcare, Wauwatosa, WI) and an Image Quant LAS 4000 (GE Healthcare Life Sciences, UK).

Statistical Analysis

Differences between two groups were estimated with Student's *t*-tests, χ^2 analysis, and analysis of variance (ANOVA). Survival curves

were generated according to the Kaplan–Meier method. The differences between survival curves were examined by using the log-rank test. In addition, univariate and multivariate survival analyses were performed by using the Cox proportional hazards model. A result was considered statistically significant when the relevant P-value was less than 0.05. All statistical analyses were performed with JMP 5.0 software (SAS Institute, Inc., Cary, NC).

RESULTS

SIRT6 Immunohistochemical Staining in Clinical NSCLC Samples

First, we investigated SIRT6 expression in 98 NSCLC samples by immunohistochemistry. Representative immunohistochemical images of different expression levels of SIRT6 are shown in Figure 1. Our data demonstrated that SIRT6 was expressed at higher levels in cancer tissues than in the corresponding noncancerous tissues. Additionally, SIRT6 expression was predominantly observed in the cytoplasm. Fifty-five (56.1%) NSCLC specimens were assigned to the cytoplasmic high-SIRT6 expression group, and 43 (43.8%) were assigned to the cytoplasmic low-SIRT6 expression group.

Association Between SIRT6 Expression and Clinicopathological Factors of NSCLC

Next, we evaluated SIRT6 expression in the cytoplasm and nucleus of cells within the set of NSCLC tissues and analyzed the correlation between SIRT6 cellular localization and clinicopathological factors and prognosis. The associations between cytoplasmic SIRT6 expression in the NSCLC specimens and ten clinicopathological characteristics (i.e., gender, age, smoking status, histological type, T factor, lymph node metastasis, pathological stage, chemotherapy after operation, radiation after operation, and recurrence) are shown in Table I. There was a significant correlation between cytoplasmic SIRT6 expression and histological type ($P = 0.0018$). However, no significant differences were observed for other factors.

Next, we examined the cytoplasmic and nuclear expression of SIRT6 simultaneously, and classified each specimen into one of two groups: specimens with cytoplasmic high-SIRT6 expression with nuclear low-SIRT6 expression (the SIRT6 cH/nL group) and specimens with other expression profiles. The relationships between cytoplasmic/nuclear expression of SIRT6 in the NSCLC specimens and the ten clinicopathological characteristics are shown in Table II. There were significant correlations between cytoplasmic/nuclear expression of SIRT6 and histological type ($P = 0.018$) and pathological staging ($P = 0.01$).

Prognostic Significance of SIRT6 Expression in Patients With NSCLC

The overall survival rates of patients with tumors that were assigned to the cytoplasmic high-SIRT6 expression group were significantly lower than those of patients with tumors that were assigned to the cytoplasmic low-SIRT6 expression group ($P = 0.0223$; Fig. 2A). In contrast, patients in the nuclear high-SIRT6 expression group tended to have better prognoses ($P = 0.0924$; Fig. 2B), but no significant difference was observed. The SIRT6 cH/nL group had significantly shorter overall survival times ($P < 0.01$) and shorter recurrence-free survival times ($P = 0.01$) than did patients with other expression profiles (Fig. 2C, D). Moreover, we analyzed overall survival rate according to pathological stage or histological type. In stage III, patients exhibiting the SIRT6 cH/nL expression profile had poorer prognoses than did other patients with $P < 0.01$, Supplementary Figure S1C. Patients with lung adenocarcinoma exhibiting the SIRT6 cH/nL expression profile had poorer prognoses than did other patients with adenocarcinoma ($P < 0.01$; Supplementary Figure S2). The evaluation of SIRT6 cH/nL was an independent prognostic factor for poor survival in multivariate analysis (Supplementary Table S1).

Regulation of Autophagy by SIRT6 was Associated With Paclitaxel Sensitivity in the Lung Adenocarcinoma Cell Line A549

Western blot analysis was used to determine the expression of SIRT6, NFκB, Beclin1 (an autophagy marker), and BCL2 (an anti-apoptotic

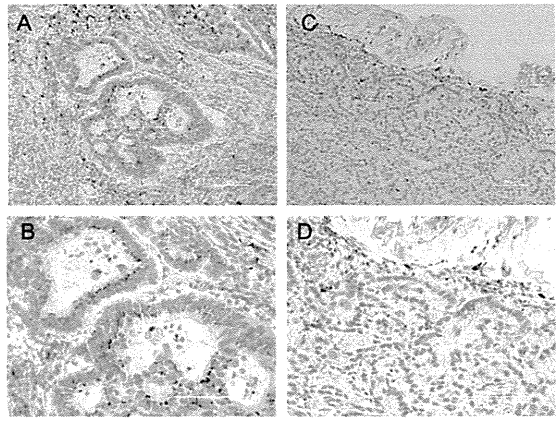

Fig. 1. Immunohistochemical staining of SIRT6 in primary NSCLC. **A**: High cytoplasmic expression and low nuclear expression of SIRT6 in a representative NSCLC sample (200× magnification). **B**: High cytoplasmic expression and low nuclear expression of SIRT6 in a representative NSCLC sample (400× magnification). **C**: Low cytoplasmic expression and high nuclear expression of SIRT6 in a representative NSCLC sample (200× magnification). **D**: Low cytoplasmic expression and high nuclear expression of SIRT6 in a representative NSCLC sample (400× magnification).

TABLE I. Relationships Between Clinicopathological Factors and SIRT6 Expression in the Cytoplasm

	Cytoplasm SIRT6		
	Low expression	High expression	
Factors	n = 43	n = 55	*P*-value
Age at operation	65.5 + 1.61	66.0 + 1.42	0.83
Gender			
Male	25	40	0.13
Female	18	15	
Smoking status			
Current, former	25	40	0.13
Never	18	15	
Histological type			
Adeno	41	40	0.0018*
Squamous	2	15	
T factor			
T1	17	25	0.14
T2	23	21	
T3	0	3	
T4	3	6	
Lymph node metastasis			
Absent	30	30	0.14
Present	13	25	
Pathological stage			
1	29	28	0.55
2	3	6	
3	9	20	
4	2	1	
Chemotherapy after operation			
Absent	23	22	0.35
Present	18	30	
Unknown	2	3	
Radiation after operation			
Absent	37	45	0.6
Present	4	7	
Unknown	2	3	
Recurrence			
Absent	29	30	0.19
Present	14	25	

*$P < 0.05$.

TABLE II. Relationships Between Clinicopathological Factors and SIRT6 Expression in the Cytoplasm/Nucleus

	Cytoplasm/Nuclear SIRT6		
	High/Low expression	Others	
Factors	n = 33	n = 65	*P*-value
Age at operation	66.1 + 1.83	65.6 + 1.30	0.8
Gender			
Male	25	40	0.15
Female	8	25	
Smoking status			
Current, former	26	39	0.05*
Never	7	26	
Histological type			
Adeno	23	58	0.018*
Squamous	10	7	
T factor			
T1	15	27	0.18
T2	11	33	
T3	2	1	
T4	5	4	
Lymph node metastasis			
Absent	16	43	0.07
Present	17	21	
Pathological stage			
1	15	42	0.01*
2	2	7	
3	16	13	
4	0	3	
Chemotherapy after operation			
Absent	13	32	0.56
Present	18	30	
Unknown	2	3	
Radiation after operation			
Absent	26	56	0.51
Present	5	6	
Unknown	2	3	
Recurrence			
Absent	15	24	0.41
Present	18	41	

*$P < 0.05$.

protein) in six human lung carcinoma cell lines, that is, A549, H1975, H2009, EBC-1, and RERF-LC-AI. Of the three adenocarcinoma cell lines, SIRT6 was highly expressed only in A549 cells. In contrast, both of the two squamous cell carcinoma cell lines showed high SIRT6 expression levels (Fig. 3A). Moreover, A549 cells exhibited accumulation of both NFκB and Beclin 1. However, the expression levels of these two proteins were low in lung squamous cell carcinoma cell lines (Fig. 3A).

To investigate the function of SIRT6 in NSCLC cells, A549 and EBC-1 cells were transfected with *SIRT6* siRNA. Reduced expression of SIRT6 protein was confirmed in both A549 and EBC-1 cells (Fig. 3B). The expression levels of NFκB and Beclin1 in A549 cells were decreased in cells transfected with *SIRT6* siRNA compared to control cells, whereas no remarkable changes were observed in EBC-1 cells. BCL2 expression levels were not altered in A549 or EBC-1 cells (Fig. 3B). These data confirmed that *SIRT6* siRNA was effective at knocking down SIRT6 expression.

Next, we examined the effects of SIRT6 knockdown on cell proliferation and paclitaxel sensitivity in lung cancer cells. Interestingly, knockdown of SIRT6 did not affect the proliferation of A549 or EBC-1 cells (Supplementary Figure S3). However, in A549 cells, knockdown of SIRT6 improved cell sensitivity to paclitaxel (Fig. 3C, D). These data confirmed the role of SIRT6 in mediating drug sensitivity in patients with lung cancer.

To confirm these results, we analyzed the correlation between prognosis and SIRT6 expression profiles in patients who had received chemotherapy. We found that SIRT6 cH/nL patients who had been given chemotherapeutic agents in the adjuvant and/or recurrent settings tended to show poorer prognoses than did patients with other SIRT6 expression profiles ($P = 0.05$; Fig. 4). The evaluation of SIRT6 cH/nL was an independent prognostic factor for poor survival in multivariate analysis (Supplementary Table S2).

DISCUSSION

In this study, we showed that the high expression of SIRT6 in the cytoplasm in primary cancer tissues from patients with NSCLC was associated with poor prognosis. Moreover, patients with high cytoplasmic expression and low nuclear expression of SIRT6 exhibited poorer prognosis than did patients with other SIRT6 expression profiles. Moreover, in our in vitro analysis of the effects of SIRT6 knockdown, we found that paclitaxel sensitivity was improved in A549 cells, but not EBC-1 cells, transfected with *SIRT6* siRNA.

SIRT6 has been reported to be predominantly localized in the nucleus [5]. However, in the present study, we found that SIRT6 was mainly present in the cytoplasm in NSCLC. Histone deacetylation activity by nuclear SIRT6 inhibits the expression of Hif1α and NFκB, both of which are associated with chemotherapy resistance [13,14]. On the other hand, cytoplasmic SIRT6 coexists with spindle fibers in the S phase of the cell cycle;[15] therefore, cytoplasmic SIRT6 may reflect the cell cycle regulatory function of SIRT6. From these observations, we hypothesize that intracellular localization of SIRT6, as measured by immunohistochemical staining, could be a useful marker of chemosensitivity and cell cycle progression in patients with NSCLC.

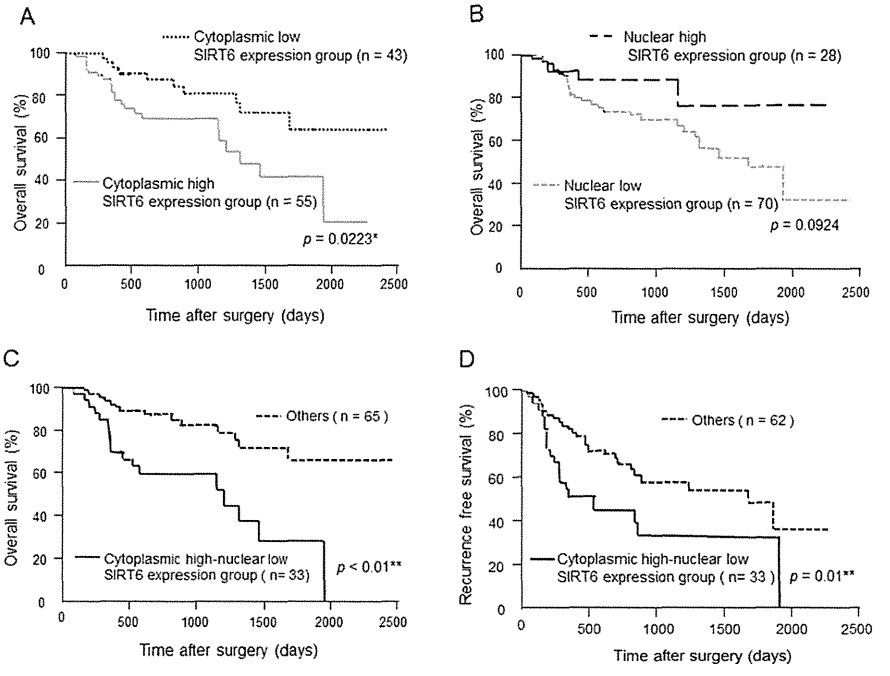

Fig. 2. Kaplan–Meier curves for patients with NSCLC according to the level of SIRT6 expression. **A**: Overall survival curves for patients with NSCLC (n = 98) based on the level of cytoplasm SIRT6 expression ($P = 0.0223$). **B**: Overall survival curves for patients with NSCLC based on the level of SIRT6 expression in the nucleus ($P = 0.0924$). **C**: Overall survival curves for patients with NSCLC based on the level of SIRT6 expression in the cytoplasm and nucleus ($P < 0.01$). **D**: Recurrence-free survival curves for patients with NSCLC based on the level of SIRT6 expression in the cytoplasm and nucleus ($P = 0.01$).

In this study, we examined SIRT6 expression in three lung adenocarcinoma cell lines; of these cell lines, only A549 cells expressed SIRT6. However, SIRT6 expression was high in both EBC-1 and RERF-LC-AI cells, which are derived from squamous cell carcinoma. Similarly, in NSCLC clinical specimens, high expression of cytoplasmic SIRT6 was frequently observed in squamous cell carcinoma samples. The high rate of genomic loss of 19p13.3, the location of the *SIRT6* gene, has been reported in patients with lung adenocarcinoma [16]. Moreover, array CGH data from patients with NSCLC showed higher rates of 19p13.3 gains in patients with squamous cell carcinoma than in patients with adenocarcinoma [17]. According to these reports, one of the reasons that patients with adenocarcinoma and squamous cell carcinoma exhibit different levels of SIRT6 expression may be due to such genomic alterations.

Previous studies have reported that SIRT6 regulates cellular sensitivity to chemotherapy by controlling BCL2 expression in prostate cancer cells [11]. However, we could not validate the association between SIRT6 and BCL2 expression in our in vitro SIRT6 suppression analysis. Therefore, we focused on analyzing the relationship between SIRT6 and autophagy, a key mechanism mediating chemotherapy resistance [7]. SIRT6 suppression has also been shown to inhibit the induction of autophagy via regulation of mTOR signaling [6]. Furthermore, induction of autophagy has been reported to be necessary for activation of NFκB signaling [18], which is known to be associated with cancer progression and chemoresistance. Indeed, in our study, A549 lung adenocarcinoma cells expressing Beclin1 and NFκB showed improved paclitaxel sensitivity in SIRT6-knockdown cells. However, this change was not observed in EBC-1 cells, a lung squamous cell carcinoma cell line, because of the absence of NFκB and the autophagy maker Beclin1. Therefore, we hypothesized that the regulation of autophagy by SIRT6 may enhance paclitaxel sensitivity only in A549 cells, which may utilize autophagy for cell survival and chemoresistance. Thus, targeting SIRT6 may be useful for the treatment of lung adenocarcinoma patients with paclitaxel-resistant tumors via induction of autophagy and activation of NFκB signaling. Further studies are needed to determine the clinical relevance of SIRT6 in lung squamous cell carcinoma.

To target SIRT6 in NSCLC patients, using the small interfering RNA or microRNA of SIRT6 for recovery of chemo resistance in SiRT6 high expressing patients. Actually, small RNAs, including microRNAs, have attracted attention as potential new tools for cancer therapeutic strategies [19,20]. Moreover, it is required that evaluation of cross activation of preexisting SIRT inhibitors for SIRT6, and screening of new candidate drugs as the inhibitor of SIRT6.

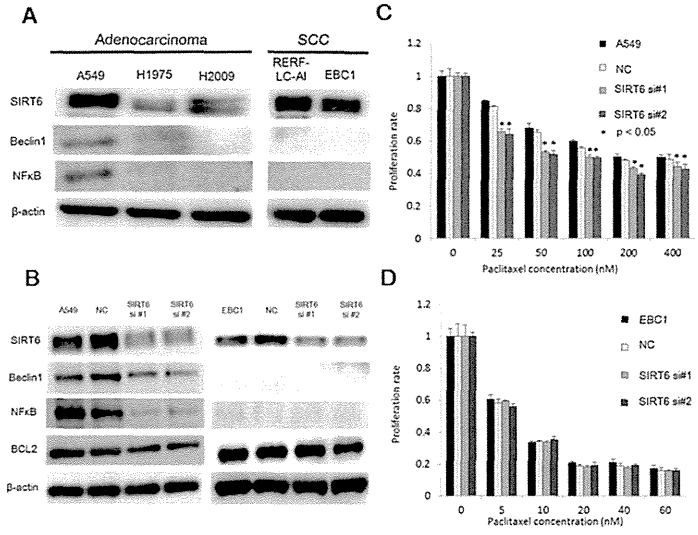

Fig. 3. Functional analysis of human NSCLC cell lines transfected with SIRT6 siRNA. **A**: Protein expression levels of SIRT6, the autophagy marker Beclin1, NFκB, and β-actin were examined in the adenocarcinoma cell lines A549, H1975, and H2009 in the squamous cell carcinoma cell lines RERF-LC-AI and EBC1 were measured by Western blotting. β-Actin was used as the loading control. **B**: Protein expression levels of SIRT6, Beclin1, NFκB, and BCL2 in A549 and EBC1 cells transfected with SIRT6 siRNA were measured by Western blotting. β-Actin was used as the loading control. N–C: negative control. **C**: A WST assay was performed to evaluate the paclitaxel sensitivity of A549 cells transfected with SIRT6 siRNA. N–C: negative control ($^*P < 0.05$). **D**: A WST assay was performed to evaluate the paclitaxel sensitivity of EBC1 cells transfected with SIRT6 siRNA. N–C: negative control.

In conclusion, the predominantly cytoplasmic localization of SIRT6 expression was correlated with poor prognosis and reduced chemosensitivity in patients with NSCLC. SIRT6 expression and localization could be a useful prognostic marker for patients with NSCLC. In our in vitro *SIRT6* siRNA analysis, we found that SIRT6 could regulate paclitaxel sensitivity via induction of autophagy. Therefore, during the development of new molecular cancer therapies, SIRT6 may be a promising candidate for targeting chemoresistance in lung adenocarcinoma.

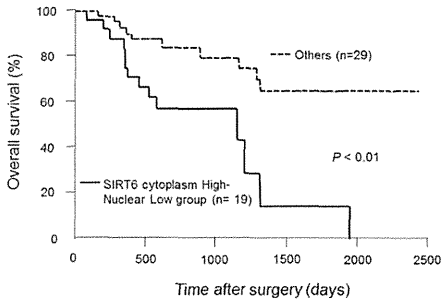

Fig. 4. Kaplan–Meier curves according to the level and localization of SIRT6 expression in patients with NSCLC treated with postoperative chemotherapy. Patients received chemotherapy in the adjuvant and recurrent settings. Curves were constructed to show differences between patients with high cytoplasmic and low nuclear expression of SIRT6 versus patients with other SIRT6 expression profiles ($n = 19$ vs. $n = 29$, respectively; $P < 0.01$).

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RESEARCH

Expression of Amino Acid Transporters (LAT1 and ASCT2) in Patients with Stage III/IV Laryngeal Squamous Cell Carcinoma

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Abstract The aim of this study is to evaluate the clinicopathological significance of L-type amino acid transporter 1 (LAT1) expression in patients with advanced laryngeal squamous cell carcinoma (LSCC). A total of 73 patients with advanced LSCC were retrospectively reviewed. Tumor sections were stained by immunohistochemistry for LAT1, 4F2hc, system ASC amino acid transporter-2 (ASCT2), cell proliferation by Ki-67, microvessel density (MVD) determined by CD34 and p53. A positive LAT1, 4F2hc and ASCT2 expression (staining more than a quarter) in the primary sites were recog-

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nized in 85, 80 and 45 %, respectively, and a high LAT1, 4F2hc and ASCT2 expression (staining more than a half) yielded 48, 31 and 18 %, respectively. High expression of LAT1 was significantly associated with lymph node metastasis, 4F2hc, ASCT2, Ki-67 and p53. The expression of LAT1 was significantly correlated with ASCT2, 4F2hc, cell proliferation, and MVD. By univariate analysis, there was no statistically significant relationship between LAT1 expression and prognosis in advanced LSCC. LAT1, 4F2hc and ASCT2 were highly expressed in patients with advanced laryngeal cancer. Our study suggests that the expression of LAT1 plays a crucial role in the metastasis and tumor progression in advanced LSCC.

Keywords Larynx · LAT1 · CD98 · Imunohistochemistry · ASCT2 · Prognostic factor

Introduction

Head and neck cancer is a malignant tumor arising from a different site in the upper aerodigestive tract, and the most common sites include the larynx, the oropharynx, the hypopharynx, and the oral cavity [1]. Surgery plus sequential chemoradiotherapy is generally considered as an appropriate treatment for patients with locally advanced diseases [2]. Laryngeal squamous cell carcinoma (LSCC) is the second most common malignant tumor of the head and neck cancers, and the occurrence of LSCC is believed to be associated with tobacco use, alcohol consumption, sex, air pollution and occupational factors [1, 2]. But, LSCC is an aggressive malignant disease with dismal prognosis, and the patients with LSCC need a promising therapeutic strategy for improving their outcome. The prognostic factors to improve the prognosis after treatment are currently still controversial. Therefore, a reliable biomarker should be established for improving the therapeutic efficacy.

Amino acid transporters play an essential role in the growth, proliferation and survival of both normal cells and transformed cells [3]. Of these transporters, L-type amino acid transporter 1 (LAT1) and system ASC amino acid transporter-2 (ASCT2) are shown to be significantly linked to the pathogenesis of cancer cells [4, 5]. LAT1 is an L-type amino acid transporter that transports large neutral amino acids, such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine, and requires a covalent association with the heavy chain of 4 F2 cell surface antigen (4F2hc) for its functional expression in the plasma membrane [5, 6]. LAT1 has been closely related to cancerous or proliferative cells and is highly expressed in the proliferating tissues [4–6]. Several in vitro studies have implicated that a LAT1 inhibitor could be an effective therapeutic option for patients with human neoplasms [7–9]. ASCT2 is a Na^+-dependent transporter responsible for the transport of neutral amino acids, including glutamine, leucine and isoleucine [10]. ASCT2 is a major glutamine transporter and is associated with tumor growth and proliferation in cancer cells [4, 11]. By several studies, both LAT1 and ASCT2 are shown to play crucial roles in the development and progression of various human neoplasms and are significant biomarkers for predicting worse outcome [7, 12–23]. But, the clinicopathological significance of amino acid transporters such as LAT1 and ASCT2 expression remains unknown in patients with LSCC. In the present study, we focused on the protein expression of LAT1 in the resected tissue specimens, and the expression of LAT1 and ASCT2 were correlated with cell proliferation and angiogenesis.

Material and Methods

Patients

We analyzed 80 consecutive patients with pathologically confirmed LSCC who underwent surgical resection at Gunma University Hospital and Gunma Prefectural Cancer Center between 2001 and 2012. Five patients had pathological stage I or II disease. The specimens of two patients were not available. Patients who had received primary chemotherapy and/or radiotherapy were excluded from the study. Therefore, a total of 73 patients with stage III/IV disease were analyzed. Formalin-fixed, paraffin-embedded tumor samples from 73 patients for the primary manifestation of LSCC were examined. All surgical specimens were reviewed and classified according to the WHO classification by the two experienced pathologists who were unaware of clinical or imaging findings. Pathologic tumour-node-metastasis (TNM) stages were established using the Classification of Malignant Tumours by the International Union against Cancer (UICC) and American Joint Committee on Cancer (AJCC) system. The study protocol was approved by the institutional review board.

Immunohistochemical Staining

LAT1 expression was determined by immunohistochemical staining with an anti-human LAT1 antibody (1.6 mg/mL, anti-human monoclonal mouse IgG_1, KM3149, provided by Kyowa Hakko Co., Ltd.; dilution of 1:800). The specificity of the antibody was confirmed by Western blotting as shown in Supplemental Fig. S1. A specific single band was detected with the antibody in HEK293 cells stably expressing human

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Table 1 Patient's demographics according to LAT1 expression

	Total	LAT1		
Variable		High	Low	*p*-value
	(*n*=73)	(*n*=35)	(*n*=38)	
Age				
≤65 years / >65 years	19 / 54	11 / 24	8 / 30	0.424
Sex				
Male / Female	69 / 4	34 / 1	35 / 3	0.615
Differentiation				
WD or MD / PD	25 / 48	11 / 24	14 / 24	0.805
Stage				
III / IV	22 / 51	10 / 25	12 / 26	0.804
Primary tumor status				
T1-3 / T4	37 / 36	19 / 16	18 / 20	0.641
Lymph node status				
N0 / N1-3	32 / 41	20 / 15	12 / 26	**0.035**
Primary site				
Supraglottic / Glottic	40 / 33	19 / 16	21 / 17	>0.999
Lymphatic permeation				
Positive / Negative	55 / 18	26 / 9	29 / 9	>0.999
Vascular invasion				
Positive / Negative	48 / 25	26 / 9	22 / 16	0.216
Resected status				
Negative / Positive	68 / 5	31 / 4	37 / 1	0.187
Adjuvant chemotherapy				
Yes / No	44 / 29	25 / 10	19 / 19	0.093
4F2hc				
High / Low	23 / 50	16 / 19	7 / 31	**0.022**
ASCT2				
Positive / Negative	33 / 40	21 / 14	12 / 26	**0.019**
Ki-67				
High / Low	21 / 52	15 / 20	6 / 32	**0.018**
CD34				
High / Low	36 / 37	18 / 17	18 / 20	0.816
p53				
Positive / Negative	30 / 43	21 / 14	9 / 29	**0.002**

Abbreviation: *LAT1* L-type amino acid transporter 1, *WD* well differentiated, *MD* moderate differentiated, *PD* poorly differentiated, *ASCT2* ASC-amino acid transporter 2

LAT1 but not in cells stably expressing human LAT2 (Supplemental Fig. S1). An oligopeptide (RDSKGLAAAEPTAN) corresponding to amino acid residues 7–20 of a rabbit polyclonal antibody against ASCT2 (1:300 dilution) was synthesized. The N-terminal cysteine residue was used for conjugation with keyhole limpet hemocyanin. An anti-peptide antibody was produced as described elsewhere [20]. The anti-CD98 antibody used is an affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.; 1:100 dilution) raised against a peptide mapping to the carboxy terminus of human CD98. The detailed protocol for immunostaining has been published elsewhere [18–20]. The LAT1 and CD98 expression scores were defined by the extent of staining as follows: 1, ≤ 10 % of tumor area stained; 2, 11–25 % stained; 3, 26–50 % stained; 4, 51–75 % stained; and 5, ≥76 %. The tumors in which the stained tumor cells were scored as 3, 4, or 5 were defined as positive; tumors scoring 4 or 5 were defined as having high expression. The immunohistochemical staining for CD34, Ki-67, and p53 was performed according to the procedures described in the previous reports [18–20]. The following antibodies were used: mouse monoclonal antibodies against CD34 (Nichirei, Tokyo, Japan; 1:800 dilution), Ki-67 (Dako, Glostrup, Denmark; 1:40 dilution), and p53 (D07; Dako; 1:50 dilution). The number of CD34-positive vessels was counted in four selected hot spots in a 400x field (field area of 0.26 mm^2). MVD was defined as the mean count of microvessels per 0.26 mm^2 field area. The median numbers of CD34-positive vessels were evaluated, and the tumors in which the stained tumor cells comprised more than each median value were defined as positive.

For p53, a microscopic examination of the nuclear reaction product was performed and scored. Based on previous reports [18–20], p53 expression greater than 10 % of the tumor cells was defined as positive expression. A highly cellular area of the immunostained sections was evaluated for Ki-67, and the epithelial cells with nuclear staining of any intensity were

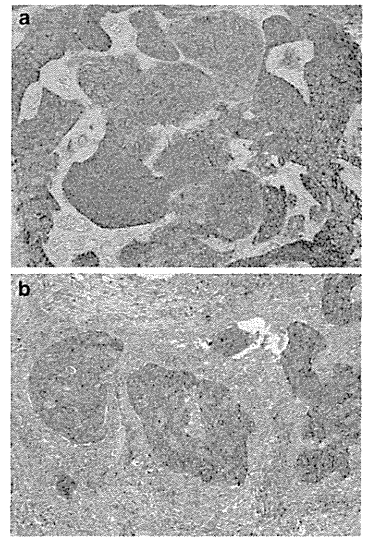

Fig. 1 Representative immunohistochemical staining of patient with tongue squamous cell carcinoma. Both LAT1 (**a**) and ASCT2 (**b**) immunostaining displays a membranous staining pattern

Table 2 Correlation between LAT1 and ASCT2, and other biomarkers

Biomarkers	Spearman γ	95 % CI	*p*-value
LAT1			
ASCT2	0.309	0.079 to 0.507	**0.007**
4F2hc	0.441	0.229 to 0.613	**<0.001**
CD34	0.056	−0.181 to 0.287	0.632
Ki-67	0.326	0.098 to 0.521	**0.005**
ASCT2			
4F2hc	0.279	0.047 to 0.482	**0.016**
CD34	−0.179	−0.397 to 0.058	0.127
Ki-67	0.007	−0.228 to 0.242	0.949

Abbreviation: *LAT1* L-type amino acid transporter 1, *ASCT2* ASC-amino acid transporter 2, *95% CI* 95 % confidence interval

defined as positive. Approximately 1000 nuclei were counted on each slide, and the proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample. The median Ki-67 labeling index value was determined, and high expression in the tumor cells was defined as a value greater than the median. The sections were assessed using light microscopy in a blinded fashion by two experienced authors.

Statistical Analysis

Probability values of <0.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association of two categorical variables. The correlation between different variables was analyzed using the nonparametric Spearman's rank test. Follow-up for these 143 patients was conducted using the patient medical records. The Kaplan-Meier method was used to estimate survival as a function of

Table 3 Univariate analysis in overall survival and progression-free survival

Variables	Overall survival		Progression-free survival	
	5-years survival rate (%)	*p*-value	5-years survival rate (%)	*p*-value
Age				
≤65 years / >65 years	42 / 43	0.686	67 / 44	0.237
Sex				
Male / Female	47 / 37	0.697	49 / 50	0.665
Disease stage				
III / IV	68 / 32	**0.031**	62 / 41	0.189
Differentiation				
WD or MD / PD	32 / 51	0.214	38 / 53	0.375
Lymphatic permeation				
Positive/Negative	35 / 80	0.021	45 / 62	0.150
Vascular invasion				
Positive/Negative	48 / 39	0.399	52 / 42	0.559
Lymph node metastasis				
Yes / No	31 / 62	0.067	35 / 65	0.155
Resected status				
R0 / R1	48 / 0	0.097	53 / 37	0.411
LAT1				
High / Low	56 / 36	0.173	58 / 41	0.408
ASCT2				
Positive/Negative	44 / 43	0.309	61 / 39	0.113
4F2hc				
High / Low	29 / 45	0.718	39 / 47	0.588
Ki-67				
High / Low	14 / 48	0.713	0 / 53	0.266
CD34				
High / Low	49 / 39	0.745	55 / 43	0.650
p53				
Positive/Negative	57 / 37	0.607	55 / 45	0.901

Abbreviation: *LAT1* L-type amino acid transporter1, *ASCT2* ASC-amino acid transporter 2

time, and survival differences were analyzed by the log-rank test. Overall survival (OS) was determined as the time from tumour resection to death from any cause. Progression-free survival (PFS) was defined as the time between tumour resection and the first disease progression or death. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using GraphPad Prism 4 software (Graph Pad Software, San Diego, CA, USA) and JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Patient's Demographics and Immunohistochemical Analysis

Table 1 shows a patient's characteristics according to the LAT1 expression. Age of the patients ranged from 50 to 88 years, and the median age was 69 years. Twenty-two patients (30 %) had stage III and fifty-one patients (70 %) stage IV. Forty-four patients (60 %) received postoperative adjuvant chemotherapy with cisplatin, 5-FU (fluorouracil), docetaxel, or S-1 (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan), oral administration of tegafur (a fluorouracil derivative drug), and/or radiotherapy. High expression of LAT1 was significantly associated with lymph node status, 4F2hc, ASCT2, Ki-67 and p53.

We performed an immunohistochemical staining on the 73 primary sites. Figure 1 shows the representative images of the immunohistochemical staining. Expression of LAT1 and ASCT2was predominantly localized at the plasma membrane of the carcinoma cells in the tumor tissues. Positive LAT1, 4F2hc and ASCT2 expression were found in 85 % (62/73), 80 % (59/73) and 45 % (33/73), respectively. Moreover, high LAT1, 4F2hc and ASCT2 expression were found in 48 % (35/73), 31 % (23/73) and 18 % (12/73), respectively. The positive expression rate of LAT1 was significantly higher than that of ASCT2 ($p<0.001$), but there was no significant difference between LAT1 and 4F2hc ($p=0.66$). The average scores of LAT1, 4F2hc and ASCT2 was 3.4±0.9, 3.1±0.9, and 2.3±1.0, respectively, and the score of LAT1 was higher than that of ASCT2 ($p<0.001$). However, no statistically significant difference was observed between the average scores of LAT1 and 4F2hc ($p=0.103$). The median number of CD34-positive vessels was 13 (range, 2 to 30), and this value was chosen as a cutoff point. The median value of the Ki-67 labeling index was 28 (range, 2 to 81), and the value of 28 % was chosen as a cutoff point. Positive p53 expression was found in 41 % (30/73) (Table 2).

Correlation Between LAT1 / ASCT2 Expression and Different Variables

We analyzed any correlation between amino acid transporters (LAT1 and ASCT2) and the other biomarkers. The expression of LAT1 was significantly correlated with ASCT2, 4F2hc, cell proliferation and MVD determined by CD34. ASCT2 expression yielded a statistically significant correlation with 4F2hc (Table 2)

Univariate and Multivariate Survival Analysis

All patients revealed a median survival time (MST) and 5-year survival rate for OS of 917 days and 42 %, respectively, and the MST and 5-year survival rate for PFS were 840 days and 43 %, respectively. Table 3 shows results of the univariate and multivariate analysis in all patients with laryngeal cancer. By the univariate analysis, only the disease stage was significantly associated with poor OS, but we could not find any factors predicting poor PFS (Fig. 2). Multivariate analysis demonstrated that disease stage was an independent and significant factor for predicting poor outcome.

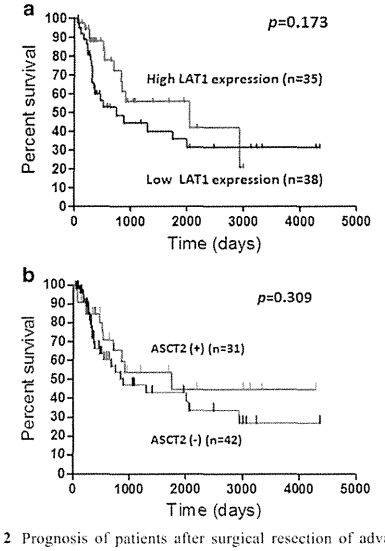

Fig. 2 Prognosis of patients after surgical resection of advanced laryngeal cancer shown by Kaplan-Meier analysis of overall survival (OS) according to the LAT1 and ASCT2 expression. A statistically significant difference was observed between the patients with high and low LAT1 expression (a) and also between the patients with high and low ASCT2 expression

Discussion

We had recently reported that the LAT1 expression of was correlated with malignant features such as vascular invasion and lymph node metastases in a variety of human neoplasms [7, 12–19]. Although the expression status was significantly different according to the histological types of cancer, its mechanism was remained to be detailed [7, 12–19]. In patients with lung cancer, positive LAT1 expression was significantly higher in squamous cell carcinoma (SQC) (91 %) than in adenocarcinoma (AC) (29 %). The patients with SQC of esophagus or oral cavity also yielded a positive LAT1 expression rate of approximately 70–90 %, whereas, a positive LAT1 expression was observed in approximately 30–60 % of patients with AC such as pancreatic cancer, biliary tract cancer and breast cancer [7, 12–19, 24]. LAT1 requires a corporative expression of 4F2hc to function as an amino acid transporter [13, 19]. In a variety of human neoplasms, LAT1 plays a crucial role in the development and progression of tumor cells, and the previous clinicopathological data indicated that LAT1 expression was closely associated with cell proliferation and angiogenesis [13, 19]. Clinical significance of LAT1 has been described to be an independent and significant marker for predicting poor survival after treatment [7, 12–19, 24]. In the present study, we found that LAT1 was highly expressed in patients with advanced laryngeal cancer and yielded a significant correlation with 4F2hc, ASCT2, cell proliferation and angiogenesis. But, no statistically significant difference in the prognosis was recognized between high and low LAT1 expression. The results of our study suggest that LAT1 has a significant malignant feature associated with tumor cell progression and metastasis for patients with advanced laryngeal SQC.

In the present study, LAT1 and 4F2hc were positively expressed in 85 and 80 % of laryngeal SQC, respectively. These expressions were closely correlated, although the positive expression of ASCT2 (45 %) was significantly lower than that of LAT1. By the recent research, both LAT1 and ASCT2 are cooperatively expressed in primary human cancers and several cell lines, and these amino acid transporters are shown to play essential role in the cell growth and survival [19–21]. Additionally, the recent studies had documented that the protein expression of LAT1 and ASCT2 could be an independent and negative prognostic biomarker in various human neoplasms [19–21]. Both LAT1 and ASCT2 expressions have a prognostic significance in cancer patients with a histology of adenocarcinoma [13, 19–21], although these amino acid transporters yielded a higher expression in SQC compared to AC. The prognosis may not be predicted according to the expression level of these transporters in patients with SQC. Our study also indicates that the expression level of LAT1 and ASCT2 may not exactly predict a survival after surgery in the advanced laryngeal SQC. Moreover, the previous studies showed that the expression level of LAT1 was closely correlated with the outcome after surgery in patients with early-stage disease, but was not correlated with the outcome in the advanced-stage disease [24, 25]. These findings were corresponding to the results of the present study. However, little is known about the detailed mechanisms of different relationship of the expression and prognosis between SQC and AC, and between early- and advanced-stage. Further study is needed to examine the prognostic role of the amino acid transporters such as LAT1 and ASCT2 according to histological types and disease stage.

LAT1 imports essential amino acids in exchange for the glutamine that has been imported by ASCT2. LAT1 and ASCT2 are cooperatively provides amino acids for protein synthesis through glutamine exchange. The present results disclosed the correlation of 4F2hc expression with the expression of both LAT1 and ASCT2. The covalent association of 4F2hc was required for LAT1 function in the plasma membrane. Amino acid such as leucine regulates cell signaling via mammalian target of rapamycin (mTOR). LAT1 and ASCT2 are closely associated with the mTOR pathway in the human tumors [4, 26, 27]. The inhibition of amino acid transporters has been shown to reduce phosphorylation of mTOR and downstream proteins by several in vitro studies [28].

In conclusion, LAT1, 4F2hc and ASCT2 were highly expressed in patients with advanced laryngeal cancer. The expression of LAT1 was closely associated with the metastasis and tumor progression, although LAT1 expression was not predictive as a prognostic factor. Further study is warranted to evaluate the prognostic significance of these amino acid transporters in the with early-stage laryngeal cancer.

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SPECIAL ARTICLE

Japan Society of Gynecologic Oncology guidelines 2015 for the treatment of ovarian cancer including primary peritoneal cancer and fallopian tube cancer

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Abstract The fourth edition of the Japan Society of Gynecologic Oncology guidelines for the treatment of ovarian cancer including primary peritoneal cancer and fallopian tube cancer was published in 2015. The guidelines contain seven chapters and six flow charts. The major changes in this new edition are as follows—(1) the format has been changed from reviews to clinical questions (CQ), and the guidelines for optimal clinical practice in Japan are now shown as 41 CQs and answers; (2) the 'flow charts' have been improved and placed near the beginning of the guidelines; (3) the 'basic points', including tumor staging, histological classification, surgical procedures, chemotherapy, and palliative care, are described before the chapter; (4) the FIGO surgical staging of ovarian cancer, fallopian tube cancer, and primary peritoneal cancer was revised in 2014 and the guideline has been revised accordingly to take the updated version of this classification into account; (5) the procedures for examination and management of hereditary breast and ovarian cancer are described; (6) information on molecular targeting therapy has been added; (7) guidelines for the treatment of recurrent cancer based on tumor markers alone are described, as well as guidelines for providing hormone replacement therapy after treatment.

Keywords Guideline · Ovarian cancer · Primary peritoneal cancer · Fallopian tube cancer · Japan Society of Gynecologic Oncology

Introduction

The number of patients with ovarian cancer is increasing in Japan and 8,631 cases were reported in 2007 [1]. Deaths due to ovarian cancer are also increasing and 4,705 patients died of this disease in 2011 [1]. Ovarian cancer is the most

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Table 1 Criteria for evaluating the quality of evidence (levels of evidence)

Level I	Evidence from meta-analyses of multiple randomized controlled trials
Level II	Evidence from randomized controlled trials, or evidence from well-designed nonrandomized controlled trials
Level III	Evidence from well-designed quasi-experimental studies, or evidence from well-designed non-experimental descriptive studies, such as comparative studies, correlation studies, and case–control studies
Level IV	Expert committee reports and opinions, or clinical experiences of respected authorities

common cause of death among malignant tumors of the female genital tract. Tumor stage is thought to be an important prognostic factor, with stage III and IV cancer having a poor prognosis [2]. Since the ovary is a pelvic organ, an ovarian tumor may not cause any early symptoms, and approximately 40–50 % of patients with ovarian cancer have stage III or IV disease (with a poor prognosis) at the time of first presentation [3]. Thus, an important challenge is to improve the outcome of treatment in patients with advanced ovarian cancer.

In order to improve the prognosis of ovarian cancer and reduce regional differences in the management of ovarian cancer in Japan, the first edition of the guidelines for the treatment of ovarian cancer was published by the Japan Society of Gynecologic Oncology in 2004. It has since been revised several times, and the fourth edition was published in April 2015. The new guidelines include seven chapters and six flow charts. The major changes in the new edition are as follows:

(1) The format has been changed from a review format to a clinical question (CQ) format, so the guidelines for optimal clinical practice in Japan are now shown as 41 CQs and answers.
(2) The 'flow charts' have been improved and are placed near the beginning of the guideline.
(3) The 'basic points', including staging, histological classification, surgical procedures, chemotherapy, and palliative care are included before the chapter.
(4) The FIGO surgical staging of ovarian cancer, fallopian tube cancer, and primary peritoneal cancer was revised in 2014 and the guideline has been revised accordingly to take the updated version of this classification into account.
(5) Procedures for the examination and management of hereditary breast and ovarian cancer (HBOC) are described.
(6) Information on molecular targeting therapy has been added.
(7) Guidelines for the treatment of recurrent cancer based on tumor markers alone and for providing hormone replacement therapy (HRT) after treatment are described.

Chapter 1: Overview

The aims of this guideline are to describe current optimal treatment for ovarian cancer (epithelial tumors, germ cell tumors, and sex cord stromal tumors), primary peritoneal cancer, and fallopian tube cancer, to reduce differences in management between medical institutions, to improve the safety of therapy and the prognosis, to reduce the burden (physical, mental, and economic) on patients by promoting optimal treatment, and to improve communication between patients and healthcare professionals.

Much of the evidence adopted in this guideline was obtained from clinical studies performed in Europe, the USA, and Japan. However, some evidence from Europe and the USA does not apply in Japan because of differences in background factors between Europe/USA and Japan. Conversely, some treatments used widely in Japan are uncommon in Europe and the USA. In such cases, the current consensus for disease management in Japan is prioritized in this guideline.

This guideline was created according to the principles of 'evidence-based medicine', which is a standard method for producing clinical practice guidelines. The quality of evidence was evaluated using the criteria shown in Table 1 [4, 5]. In addition, the grade of each recommendation in the guideline was determined using the criteria set out in Table 2 [4–6].

Chapter 2: Epithelial ovarian cancer

Treatment of epithelial ovarian cancer is summarized as flow chart 1 (Fig. 1).

CQ 01: What is the optimal surgical procedure for ovarian cancer when the tumor seems to be localized to the ovary?

Recommendations

(1) In addition to bilateral salpingo-oophorectomy + total hysterectomy + omentectomy, peritoneal cytol-

Table 2 Grading of recommendations

Grade A	The proposed treatment is strongly recommended
	In principle, there is at least one source of Level I evidence showing efficacy of the treatment
Grade B	The proposed treatment is recommended
	In principle, there is at least one source of Level II evidence showing efficacy of the treatment
Grade C1	The proposed treatment may be considered. However, there is not enough scientific evidence
	(or the treatment may have efficacy, although sufficient scientific evidence has not been obtained)
	There are multiple sources of Level III evidence showing efficacy of the treatment and the outcomes are roughly consistent
Grade C2	There is not enough scientific evidence, and the treatment is not recommended in routine clinical practice
Grade D	The treatment is not recommended (usefulness or efficacy have not been shown, and the treatment may be harmful)

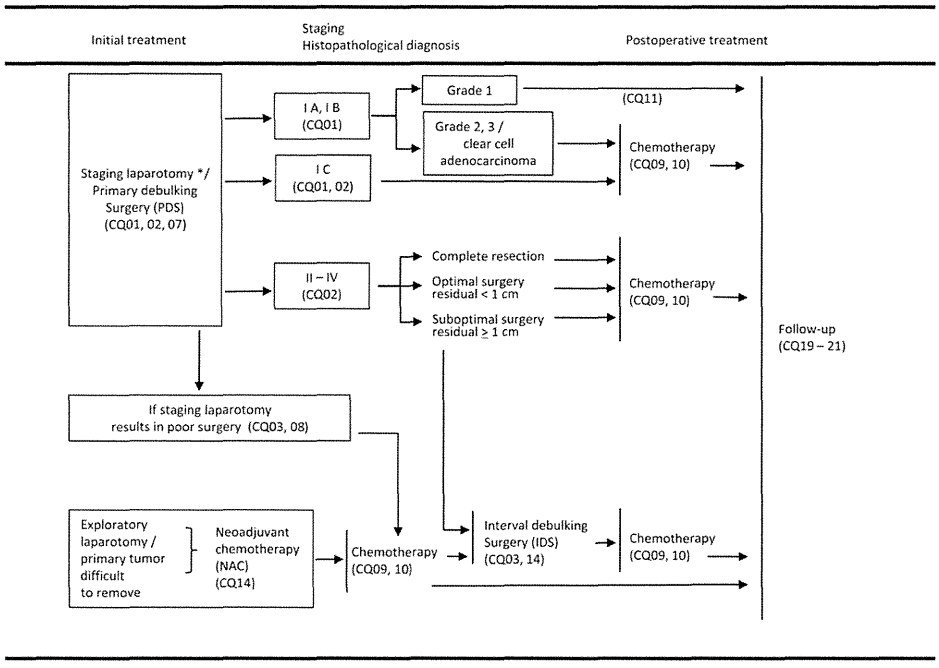

Fig. 1 Flow chart 1: treatment of epithelial ovarian cancer. *Staging laparotomy—bilateral salpingo-oophorectomy + total hysterectomy + omentectomy + peritoneal cytology + pelvic/para-aortic lymph node dissection (biopsy) + biopsies from sites in the abdominal cavity

ogy + pelvic/para-aortic lymph node dissection (biopsy) + biopsies from sites in the abdominal cavity are recommended (Grade B).

(2) When biopsies are obtained from sites in the abdominal cavity, sampling from the following sites should be considered—pouch of Douglas, parietal peritoneum, surface of the diaphragm, intestinal tract, mesentery, and suspected lesions (Grade C1).

CQ 02: What is the optimal surgical procedure for ovarian cancer that is thought to be stage II or a more advanced stage preoperatively?

Recommendations

Maximal debulking surgery to accomplish complete resection (no gross residual tumor) is strongly recommended (Grade A).

CQ 03: Is interval debulking surgery (IDS) recommended for advanced ovarian cancer if primary debulking surgery (PDS) had a suboptimal outcome?

Recommendations

As a treatment option, IDS should be considered during chemotherapy for patients with advanced cancer if previous surgery had a suboptimal outcome (Grade C1).

CQ 04: What is the optimal management if a patient wishes to preserve fertility?

Recommendations

(1) Detailed informed consent about preservation of fertility is necessary (Grade A).
(2) As the basic operative procedure to preserve fertility, affected-side salpingo-oophorectomy + omentectomy + peritoneal cytology is recommended (Grade B).
(3) In addition to the above-mentioned basic procedure, biopsy of the contralateral ovary, biopsy (dissection) of the pelvic/para-aortic lymph nodes, and biopsies from sites in the abdominal cavity should be considered as part of staging laparotomy (Grade C1).

CQ 05: Is risk-reducing salpingo-oophorectomy (RRSO) recommended for patients with the *BRCA1* or *BRCA2* gene mutation?

Recommendations

It is recommended that RRSO only be performed by a gynecologic oncologist who is a member of the Japan Society of Gynecologic Oncology in cooperation with a clinical geneticist at a medical facility with an established genetic counseling system and cooperative pathologists, after review and approval by the institutional ethics committee (Grade B).

CQ 06: Is laparoscope-assisted surgery possible?

Recommendations

(1) Currently, laparoscope-assisted surgery is not recognized as a standard procedure that can be substituted for laparotomy (Grade C2).
(2) However, in patients with advanced cancer, laparoscope-assisted surgery may be substituted for laparotomy to observe the abdominal cavity and collect tissue samples (Grade C1).

CQ 07: For which patients is intraoperative rapid pathological examination recommended?

Recommendations

For patients in whom judgment between benign/borderline malignancy/malignancy is difficult based on preoperative evaluation and intraoperative findings, intraoperative rapid pathological examination is recommended for selecting the optimal surgical procedure (Grade B).

CQ 08: What is the recommended management of a patient in whom ovarian cancer is diagnosed after surgery?

Recommendations

Staging laparotomy (re-laparotomy) is recommended (Grade B).

CQ 09: What chemotherapy regimen is recommended as first-line therapy?

Recommendations

(1) Paclitaxel + carboplatin (conventional TC therapy) is strongly recommended (Grade A).
(2) Dose-dense TC therapy is also recommended (Grade B).

CQ 10: What chemotherapy regimens other than TC therapy are recommended as first-line therapy?

Recommendations

(1) Docetaxel + carboplatin (DC therapy) is recommended (Grade B).
(2) Cisplatin monotherapy or carboplatin monotherapy can be considered (Grade C1).

CQ 11: Which patients do not need postoperative chemotherapy?

Recommendations

It can be omitted for patients with stage I A/I B, Grade 1 disease confirmed by staging laparotomy (Grade B).

CQ 12: Should first-line chemotherapy be selected by considering tumor histology?

Recommendations

This is not recommended because there is insufficient evidence to show that standard treatment should be changed depending on tumor histology (Grade C2).

CQ 13: Is intraperitoneal chemotherapy recommended as the first-line therapy?

Recommendations

Intraperitoneal chemotherapy should be considered for patients with advanced cancer who have undergone optimal surgery (Grade C1).

CQ 14: Are neoadjuvant chemotherapy (NAC) and IDS recommended for advanced ovarian cancer if optimal surgery is thought to be impossible?

Recommendations

For patients with advanced cancer in whom it is thought that primary surgery will not result in an optimal outcome, preoperative chemotherapy and debulking surgery (NAC + IDS) are recommended as a treatment option (Grade B).

CQ 15: Is maintenance chemotherapy recommended after complete remission is achieved?

Recommendations

It is not recommended, because usefulness of maintenance chemotherapy has not been demonstrated (Grade C2).

CQ 16: What management approach is recommended if complete remission is not achieved by initial treatment?

Recommendations

Additional treatment (second-line chemotherapy and radiotherapy), participation in a clinical trial, or best supportive care should be considered (Grade C1).

CQ 17: What is the recommended management of serious adverse events associated with chemotherapy?

Recommendations

Hypersensitivity reactions (HSR)

(1) Premedication should be provided because taxanes, such as paclitaxel, are associated with a risk of HSR (Grade A).
(2) When carboplatin causes HSR, premedication alone cannot reduce the risk of recurrence. Therefore, switching to another drug or desensitization therapy should be considered (Grade C1).

Gastrointestinal symptoms (nausea, diarrhea)

(1) For nausea, refer to the relevant guideline [7], and provide adequate antiemetic therapy (Grade A).
(2) For mild diarrhea, antidiarrheal agents should be administered orally. For severe diarrhea complicated by other symptoms, early aggressive treatment should be performed, such as fluid replacement and administration of an antibacterial agent (Grade A).

Myelosuppression/febrile neutropenia

Refer to the relevant guideline [8], and provide adequate treatment with an antibacterial agent and/or a granulocyte-colony stimulating factor (G-CSF) preparation (Grade A).

CQ 18: Are any molecular targeting drugs recommended as first-line therapy or as treatment for recurrence?

Recommendations

Bevacizumab should be considered in combination with chemotherapy and as subsequent maintenance therapy. However, careful patient selection and appropriate monitoring for adverse events are required when bevacizumab is used (Grade C1).

CQ 19: What is the optimal follow-up interval after treatment?

Recommendations

After the start of initial treatment,

Years 1–2: an interval of 1–3 months
Years 3–5: an interval of 3–6 months
Year 6 onward: an interval of 1 year
The above-mentioned intervals are only intended as a guide (Grade C1).

CQ 20: What examinations/tests should be performed for follow-up after treatment?

Recommendations

(1) Taking a history and performing and pelvic examination at every visit should be considered (Grade C1).
(2) Measurement of CA125, transvaginal ultrasonography, or computed tomography scanning should be considered as required (Grade C1).

CQ 21: Is intervention for recurrence recommended if the patient only has elevation of CA125 without any symptoms?

Recommendations

Early intervention in response to elevation of CA125 alone is not necessarily recommended (Grade C2).

CQ 22: Is HRT recommended?

Recommendations

After informing the patient about its merits and demerits, HRT should be considered carefully for individual patients (Grade C1).

Chapter 3: Borderline epithelial ovarian tumors

Treatment of borderline epithelial ovarian tumors is summarized as flow chart 2 (Fig. 2).

CQ 23: What is the optimal surgical procedure for borderline epithelial ovarian tumors?

Recommendations

(1) In addition to bilateral salpingo-oophorectomy + total hysterectomy + omentectomy + peritoneal cytology, detailed intra-abdominal examination is recommended (Grade B).
(2) If suspected peritoneal lesions are found by intra-abdominal examination, removing such lesions should be considered, or taking peritoneal biopsies from several sites should be considered if there are no suspected peritoneal lesions (Grade C1).
(3) For patients who wish to preserve fertility, in addition to salpingo-oophorectomy on the affected side + omentectomy + peritoneal cytology, detailed intra-abdominal examination should be considered (Grade C1).

CQ 24: What are the indications for chemotherapy and the recommended regimens?

Recommendations

For patients with gross residual tumors and patients with invasive peritoneal implants, performing postoperative chemotherapy with platinum agents and taxanes according to the treatment regimens for ovarian cancer should be considered (Grade C1).

CQ 25: What is important for follow-up after treatment of a borderline epithelial ovarian tumor?

Recommendations

In patients with borderline epithelial tumors, long-term follow-up for at least 10 years after treatment should be considered (Grade C1).

Chapter 4: Recurrent epithelial ovarian cancer

Treatment of recurrent ovarian cancer is summarized as flow chart 3 (Fig. 3).

CQ 26: What chemotherapy regimen is recommended for recurrence after a disease-free interval (DFI) of <6 months?

Recommendations

Monotherapy that avoids cross-resistance to previous treatment is recommended (Grade B).