Table 2 Significant changes in protein expression levels as judged with G-test under $p < 0.05$ for an LCNEC vs. SCLC pair. *(Continued)*

50	CO6A3	P12111	Collagen alpha-3(VI) chain precursor	5.16	2.31E-02	0.91	32	24
51	EF1A1	P68104	Elongation factor 1-alpha 1	4.72	2.98E-02	0.83	35	28
52	PDIA6	Q15084	Protein disulfide-isomerase A6 precursor	4.01	4.52E-02	0.81	31	25
53	G3P	P04406	Glyceraldehyde-3-phosphate dehydrogenase	14.21	1.64E-04	0.77	113	95
54	ENPL	P14625	Endoplasmin precursor	5.76	1.64E-02	0.66	62	56
55	TBB2A	Q13885	Tubulin beta-2A chain	5.80	1.61E-02	0.63	69	64
56	VIME	P08670	Vimentin	5.92	1.49E-02	0.60	77	73
57	HBB	P68871	Hemoglobin subunit beta	4.88	2.72E-02	-0.53	53	110
58	TBB5	P07437	Tubulin beta chain	10.89	9.64E-04	-0.63	81	179
59	TBA1A	Q71U36	Tubulin alpha-1A chain	14.38	1.49E-04	-0.67	93	211
60	TBB2B	Q9BVA1	Tubulin beta-2B chain	5.93	1.49E-02	-0.69	35	82
61	H2B1B	P33778	Histone H2B type 1-B	6.45	1.11E-02	-0.83	25	65
62	LMNB1	P20700	Lamin-B1	7.27	7.03E-03	-0.87	25	67
63	HBA	P69905	Hemoglobin subunit alpha	5.77	1.63E-02	-0.92	17	48
64	CALM	P62158	Calmodulin	3.90	4.82E-02	-1.00	9	28
65	HNRH1	P31943	Heterogeneous nuclear ribonucleoprotein H	4.36	3.67E-02	-1.01	10	31
66	NUMA1	Q14980	Nuclear mitotic apparatus protein 1	4.83	2.80E-02	-1.01	11	34
67	LAP2A	P42166	Lamina-associated polypeptide 2 isoform alpha	5.31	2.13E-02	-1.05	11	35
68	H31T	Q16695	Histone H3.1t	6.23	1.26E-02	-1.06	13	41
69	GDIA	P31150	Rab GDP dissociation inhibitor alpha	7.65	5.67E-03	-1.09	15	48
70	TBA1C	Q9BQE3	Tubulin alpha-1C chain	14.23	1.62E-04	-1.12	27	86
71	TBA1B	P68363	Tubulin alpha-1B chain	35.27	2.88E-09	-1.16	63	202
72	K1C19	P08727	Keratin, type I cytoskeletal 19	10.64	1.11E-03	-1.19	17	58
73	HSP76	P17066	Heat shock 70 kDa protein 6	6.46	1.10E-02	-1.23	9	33
74	H12	P16403	Histone H1.2	7.59	5.87E-03	-1.31	9	35
75	TBB4	P04350	Tubulin beta-4 chain	12.66	3.73E-04	-1.50	11	48
76	MOES	P26038	Moesin	4.51	3.36E-02	-1.51	3	16
77	KU70	P12956	ATP-dependent DNA helicase 2 subunit 1	22.32	2.31E-06	-1.64	16	75
78	DYHC1	Q14204	Cytoplasmic dynein 1 heavy chain 1	8.54	3.48E-03	-1.67	5	27
79	RBBP4	Q09028	Histone-binding protein RBBP4	6.58	1.03E-02	-1.74	3	19
80	PGS1	P21810	Biglycan precursor	3.99	4.59E-02	-1.81	1	10
81	ROA1L	Q32P51	Heterogeneous nuclear ribonucleoprotein A1-like protein	7.30	6.89E-03	-1.81	3	20
82	HNRPF	P52597	Heterogeneous nuclear ribonucleoprotein F	4.77	2.90E-02	-1.93	1	11
83	RUXG	P62308	Small nuclear ribonucleoprotein G	6.40	1.14E-02	-2.15	1	13
84	1433S	P31947	14-3-3 protein sigma	4.19	4.08E-02	-2.21	0	7
85	PEG10	Q86TG7	Retrotransposon-derived protein PEG10	4.19	4.08E-02	-2.21	0	7
86	CAYP1	Q13938	Calcyphosin	4.19	4.08E-02	-2.21	0	7
87	GBB1	P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	4.19	4.08E-02	-2.21	0	7
88	NCA11	P13591	Neural cell adhesion molecule 1, 140 kDa isoform precursor	4.19	4.08E-02	-2.21	0	7
89	FSCN1	Q16658	Fascin	5.11	2.38E-02	-2.37	0	8
90	ROA0	Q13151	Heterogeneous nuclear ribonucleoprotein A0	8.98	2.74E-03	-2.43	1	16
91	MDHC	P40925	Malate dehydrogenase, cytoplasmic	7.00	8.13E-03	-2.66	0	10
92	H2A1D	P20671	Histone H2A type 1-D	8.94	2.79E-03	-2.89	0	12
93	SEGN	O76038	Secretagogin	15.915	6.63E-05	-3.51	0	19
94	MAP1B	P46821	Microtubule-associated protein 1B	16.926	3.89E-05	-3.58	0	20
95	BASP	P80723	Brain acid soluble protein 1	24.067	9.30E-07	-3.99	0	27

Proteins are listed in descending order of R_{sc} values, pooled spectral counts are listed, and "_HUMAN" are removed from UniProtKG entry names.

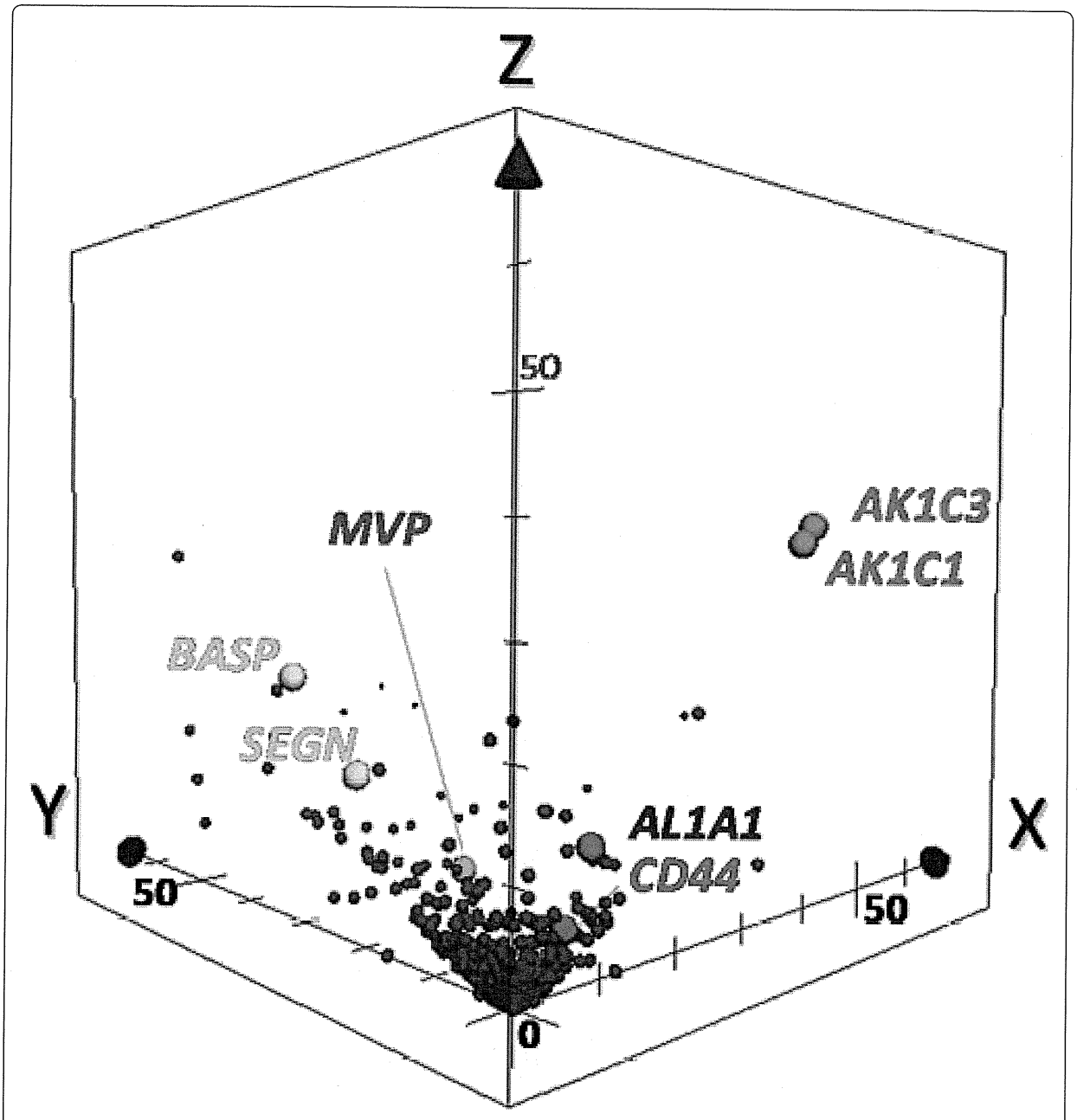

Figure 2 Marker candidates' extraction by pairwise G statistics. In the 3D scatter plot, X, Y, Z-axis shows G-values (X: LCNEC vs. LCC; Y: LCC vs. SCLC; Z: LCNEC vs. SCLC). Data point sets from 1,918 proteins were plotted with circles. AK1C1 and AK1C3 (orange), AL1A1 (purple) and CD44 (red) Proteins being located very near or on X-Z plane are isolated as candidates of specific LCNEC markers. SEGN (yellow) were located on Y-Z plane, which was already known as one of SCLC-specific markers.

were AL1A1 positive in the extent of 30 to 90%. The most intense staining (90% positive area) was observed in patient 2 of LCNEC (Table 1 and Figure 4A). On the other hand, LCC and SCLC sections with typical histology were AL1A1 negative (Figure 4A). There were four cases with weak immunoreactivity (30-80% area) which would contain the small areas mimicking some LCNEC morphology. In LCNEC four were immuno-positive (30-100% positive area) to both AK1C1 and AK1C3, and there was one more AK1C3 positive case. In LCC group one case was AK1C1 positive and four cases were AK1C3 positive; these cases showed small areas with neuroendocrine tendency in the

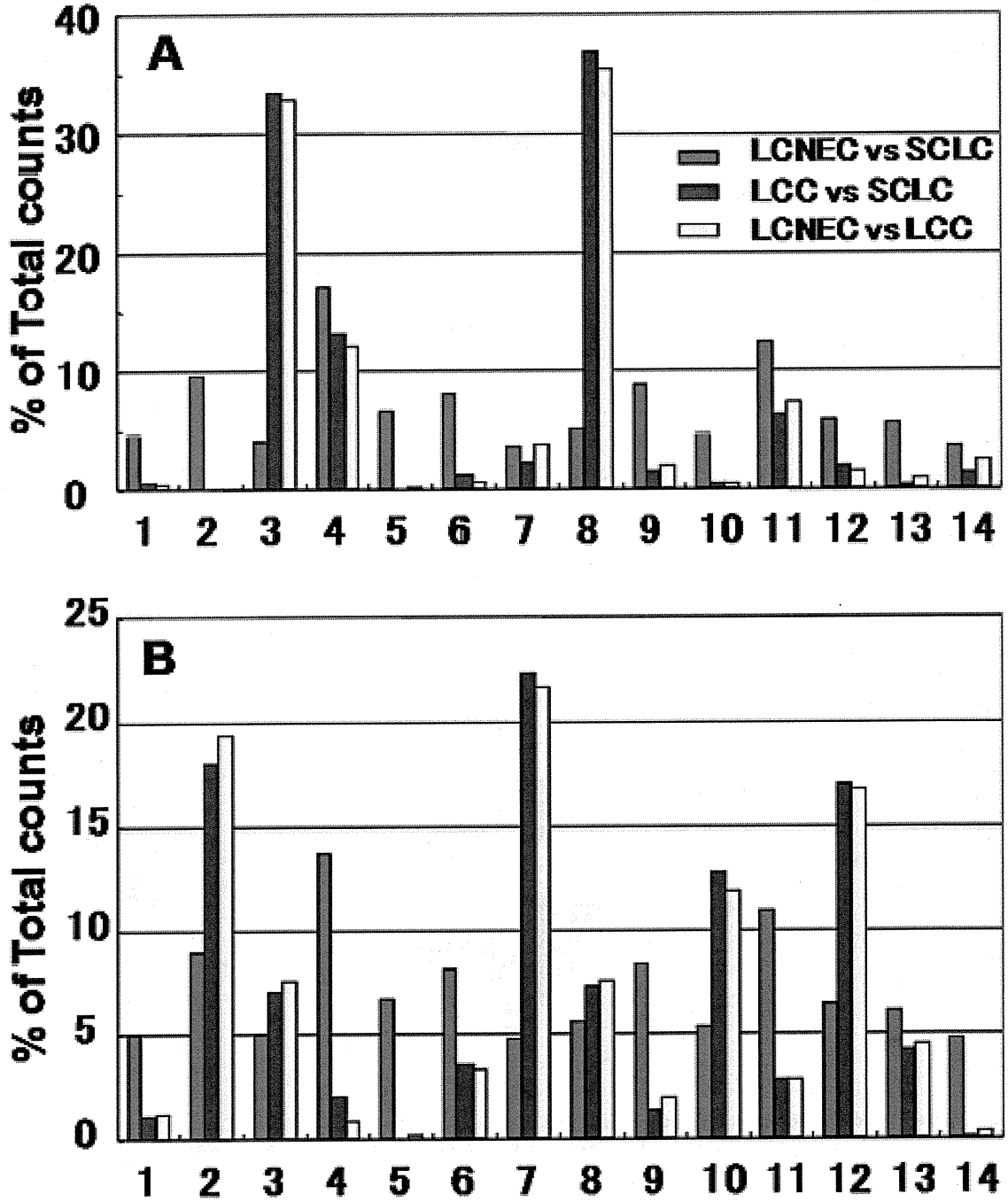

Figure 3 Gene ontology (GO) analysis on the molecular functions and cellular localization of proteins preferentially expressed in three cancer group pairs (LCNEC vs. SCLC, LCNEC vs. LCC, and LCC vs. SCLC). A) Molecular functions: 1, antioxidant activity; 2, auxiliary transport protein activity; 3, binding; 4, catalytic activity; 5, chemoattractant activity; 6, electron carrier activity; 7, enzyme regulator activity; 8, molecular function; 9, molecular transducer activity; 10, motor activity; 11, structural molecule activity; 12, transcription regulator activity; 13, translation regulator activity; 14, transporter activity. B) Cellular localizations: 1, Golgi apparatus; 2, cytoplasm; 3, cytoskeleton; 4, endoplasmic reticulum; 5, endosome; 6, extracellular region; 7, intracellular organelle; 8, membrane; 9, mitochondrion; 10, nucleus; 11, organelle membrane; 12, organelle part; 13, plasma membrane; 14, ribosome.

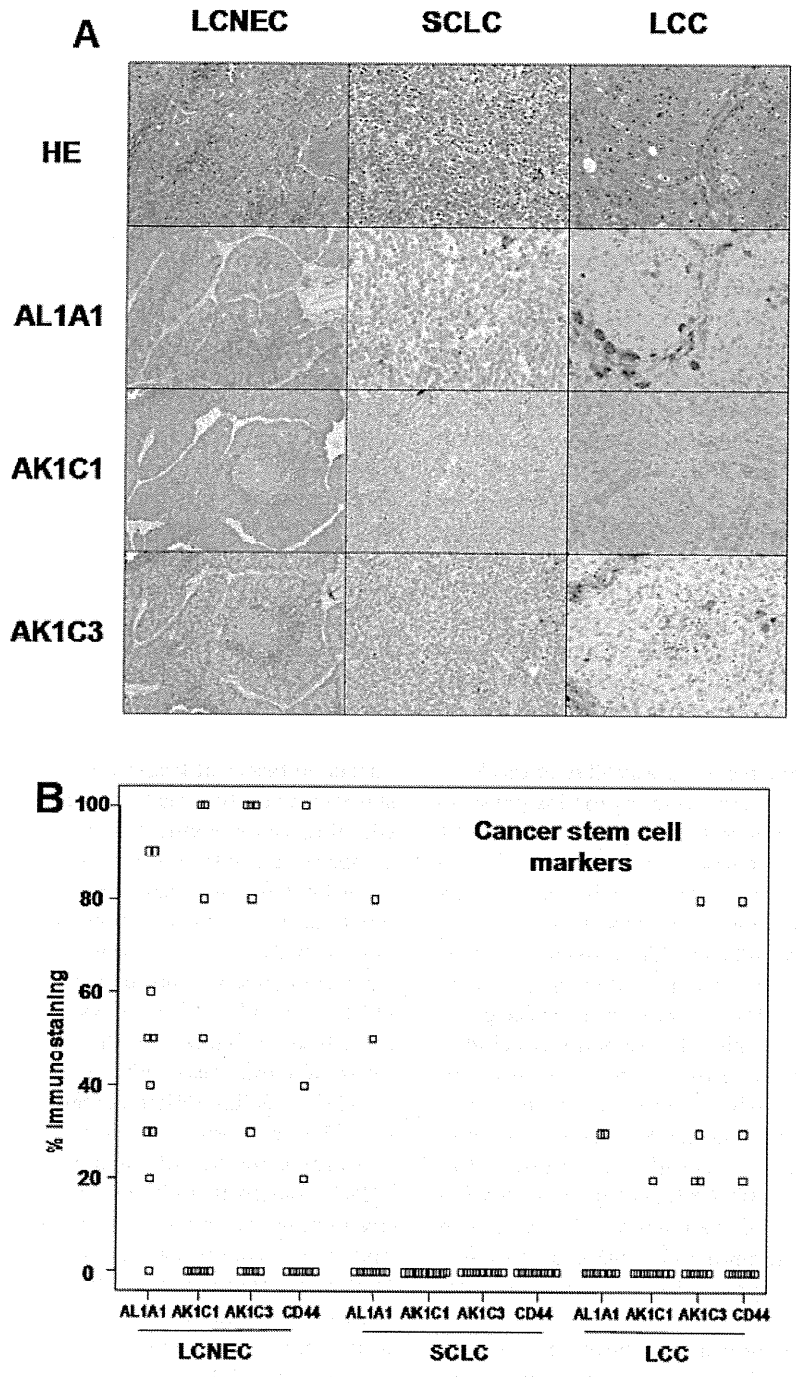

Figure 4 Imunohistochemical identification of proteomics-identifying proteins. A) Histological appearances of LCNEC, SCLC and LCC, and immunohistochemical staining of AL1A1, AK1C1 and AK1C3. Magnification, x200. B) Immunoreactivitiy with AL1A1, AK1C1, AK1C3, and CD44. The immunoreactivity was indicated as the percentage of immunopositive area at the maximal cut-surface of tumors.

tissue structure. Immnoreactivity of LCNEC cells to CD44 were the same as that of LCC.

4. Discussions

This study aimed at developing the way of proteomic distinction between LCNEC and SCLC, which will assist pathologic distinction that has not sometimes been straightforward, leading to therapeutic inefficiency. We have been focusing our attention on using laser-microdissection sampling from FFPE sections for proteomics to explore disease-related protein markers. We have already applied this method to both global semi-quantitative shotgun proteomics using spectral counting and MRM-based quantitative proteomics and successfully identified stage-related proteins on lung AC [11,12]. In this study, we used the same global shotgun method for comparison of three cancer groups (LCNEC, SCLC, and LCC) by spectral counting and explicitly interpreted three sets of pairwise G test results in the 3D G-statistic space (Figure 2). This resulted in identifying four proteins AL1A1, AK1C1 AK1C3 and CD44 that were expressed in LCNEC more than in SCLC and LCC with high probabilities. These proteomic findings using the limited scale of patients were confirmed by routine immunohistochemitry with additional patients. Moreover we identified other proteins related to these cancer groups in the present study, further demonstrating the technical feasibility of this FFPE proteomic method. The identified four proteins physiologically take part in known metabolic processes. AL1A1, AK1C1 and AK1C3 are cytosolic oxidoreductases that are involved in reduction of progesterone to the inactive form 20-alpha-hydroxy-progesterone, metabolism of steroids and prostaglandins with multi-specificity, oxidation of retinal to retinoic acid and the precursor of the storage form vitamin A, respectively. CD44 is one of cell-surface glycoproteins which relates to cell-cell interactions including adhesion and migration, and thus to tumor growth and progression [15]. When we have considered the properties common to these proteins that have apparently no functional relationship with one another, we noticed that AL1A1 [16,17], AK1C1 [18], AK1C3 [19] and CD44 [20] have been proposed to be the markers of cancer stem cells. Their expression in tumor cells could correlate with their aggressive biological behavior, drug resistance and poor prognosis, which are common characteristics of LCNEC and SCLC. The preferential expression of the cancer stem cell markers in LCNEC over SCLC suggests that the mechanism of increasing the extent of malignancy in LCNEC differs from that in SCLC. Previous studies suggested that these redox enzymes were present in a variety of malignant tumor cells. In particular, AK1C1, and AK1C3 are reported in human non-small cell lung carcinoma (A549) cells [21], and a high expression of AL1A1 in lung cancer cell lines, especially in AC cell lines compared to LCC and SCLC cell lines [22-24]. To our knowledge, however, this is the first report of the statistically significant proteomic detection of AL1A1, AK1C1 and AK1C3 in clinical samples of lung cancers, especially in LCNEC. Out of the top five LCNEC-specific proteins, brain-type FABP7 is present in highly infiltrative malignant glioma and associated with enhanced cell migratory activity and thus with poor prognosis [25], suggesting for its involvement in the aggressive nature of LCNEC. Out of the top five down-regulated LCNEC proteins compared with SCLC, BASP is a potential tumor suppressor [26], consistent with its down-regulation in LCNEC, and its specific expression in SCLC suggests that different mechanisms of tumor growth could operate between LCNEC and SCLC. Another SCLC-specific SEGN is a novel neuroendocrine marker that has a distinct expression pattern from the conventional ones used in this study, consistent with being negative in LCNEC, and with the reported rate for positive staining in SCLC (26 out of 31) [27]. The role of AL1A1 in lung cancers is still unknown, but it is recently reported that AL1A1 plays an important role in Notch pathway [28]. Though there has been no effective chemotherapy to LCNEC, Sorafenib, a tyrosine kinase inhibitor in the MAP kinase pathway, is effective to malignant tumor cells with AL1A [29]. AL1A1 would be not only cancer stem cell markers, but also an attractive target of treatment of LCNEC. In addition to statistically sorting protein expression levels by spectral counting, GO mapping of significant proteins on pairwise comparison ($p < 0.05$) provides insights into overall differences from pair and pair in their biological and molecular functions, and cellular components. Gene ontology distributions of molecular function and cellular components in neuroendocrine vs. non-neuroendocrine comparisons, i.e., LCNEC vs. LCC and SCLC vs. LCC, did not significantly differ from each other. On the other hand, those distributions in comparison within neuroendocrine groups, LCNEC vs. SCLC, differed greatly from those of the other pairs. This does encourage us to go ahead with further studies in this line and will promise to get target proteins of LCNEC eventually in future. We checked the rate of positive immuno-reaction of relevant antibodies with proteomics-identifying proteins for ten patients of each group (Figure 4B). Differences between the rates for all target proteins in LCNEC and SCLC are fully consistent with the proteomic results, confirming the specificity to LCNEC. The preferential expression of AL1A1 and AK1C1 in LCNEC over LCC was also immunochemically confirmed, and the rate of AL1A1 positive cases in LCC (20%) agreed with the previous results (25%, 1 of 4) [16]. In contrast, the positive staining rates of AK1C3 and CD44 in LCNEC and LCC were similar to each other. Close inspection of HE sections showed that the positive cases in LCC had small areas with neuroendocrine tendency in the tissue

structure as pointed out above. Almost all sections of LCC exhibited no immunoreactivity with the neuroendocrine markers used except for weak reactivity (20 or 30%) in only two cases. This suggests that the LCNEC like structure observed in small portions of LCC sections does not necessarily contain enough secretory granules, but presumably contain LCNEC specific AK1C3 and CD44. Confirmatory conclusion of this issue should await proof by electron micrographic immunohitochemistry. A previous study indicated that CD44 was expressed more in SC (97%) and AC (71%) compared to LCC (29%) and SCLC (0%) [30] in agreement with the present positive rates for LCC (30%) and SCLC (0%).

5. Conclusions

We concluded that AL1A1, AK1C1, AK1C3, and CD44 were specific for the LCNEC phenotype in relation to SCLC and LCC through proteomics of FFPE samples. They were useful targets to immunohistochemically distinguish LCNEC from SCLC and LCC. Though we need a variety of studies with more extensive experimental and clinical data to assess the precise function of these marker candidates and confirm them as real biomarkers, this proteomic analysis was effective to detect them and will be applied to other phenotype of malignancies.

Abbreviations

NSCLC: non-small cell lung carcinoma; LCNEC: large cell neuroendocrine carcinoma; LCC: large cell carcinoma; SCLC: small cell lung carcinoma; CSC: cancer stem cell; LC: liquid chromatography; MS: mass spectrometry; FFPE: formalin-fixed paraffin embedded; LMD: laser microdissection; MS/MS: tandem mass spectrometry; ISIS: in-sample internal standard; AL1A1: aldehyde dehydrogenase 1 family, member A 1; AK1C1: aldo-keto reductase family 1, member C1; AK1C3: aldo-keto reductase family 1, member C3; HE: hematoxylin-eosin

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Author details

[1]Dept. of Surgery I, Tokyo Medical University, Tokyo, Japan. [2]Diagnostic Pathology, Division, Tokyo Medical University, Tokyo, Japan. [3]Biosys Technologies, Inc., Tokyo, Japan. [4]Dept. of Structural Biology, Graduate School of Pharmaceutical Science. Hokkaido, University, Hokkaido, Japan. [5]Laboratory for Systems Biology and Medicine, RCAST, The University of Tokyo, Tokyo, Japan. [6]Dept. of Biophysics and Biochemistry, Osaka University, Graduate School of Medicine, Suita, Japan. [7]Medical ProteoScope Co., Ltd. Tokyo, Japan. [8]Hamon Center for Therapeutic Cancer Research, UT Southwestern Medical Center, Texas, USA. [9]Division of Systems Biomedical Technology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan. [10]Niizashiki Central General Hospital, Saitama, Japan. [11]Clinical Protein Science & Imaging, Dept. of Measurement Technology and Industrial Electrical Engineering, Lund University, Lund, Sweden.

Authors' contributions

MN coordinated the clinical and experimental parts of study and drafted the manuscript. TF and KF performed protein analysis through mass spectrometry. TK carried out proteomic data analysis. HT performed statistical analysis and helped to draft the manuscript. MK performed statistical analysis of G-test. YB helped us to use FFPE technique. AG suggested some important points of pathological diagnosis of LCNEC. MT offered clinical samples from patients. HO and TN pathologically diagnosed all samples independently. TO and NI supported us clinically and financially. NG supported us experimentally and financially. HK supported us clinically. GMV and TN coordinated FFPE project and assessed the results. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Prognostic Impact of Number of Resected and Involved Lymph Nodes at Complete Resection on Survival in Non-small Cell Lung Cancer

Hisashi Saji, MD, PhD, Masahiro Tsuboi, MD, PhD,* Koichi Yoshida, MD, PhD,* Yasufumi Kato, MD, PhD,* Masaharu Nomura, MD, PhD,† Jun Matsubayashi, MD, PhD,† Toshitaka Nagao, MD, PhD,† Masatoshi Kakihana, MD, PhD,* Jitsuo Usuda, MD, PhD,* Naohiro Kajiwara, MD, PhD,* Tatsuo Ohira, MD, PhD,* and Norihiko Ikeda, MD, PhD**

Background: Lymph node (LN) status is a major determinant of stage and survival in patients with lung cancer. In the 7th edition of the *TNM Classification of Malignant Tumors*, the number of involved LNs is included in the definition of pN factors in breast, stomach, esophageal, and colorectal cancer, and the pN status significantly correlates with prognosis.

Methods: We retrospectively investigated the prognostic impact of the number of resected LNs (RLNs) and involved LNs in the context of other established clinical prognostic factors, in a series of 928 consecutive patients with non-small cell lung cancer (NSCLC) who underwent complete resection at our institution between 2000 and 2007.

Results: The mean number of RLNs was 15. There was a significant difference in the total number of RLNs categorized between less than 10 and ≥10 ($p = 0.0129$). Although the incidence of LN involvement was statistically associated with poor prognosis, the largest statistically significant increase in overall survival was observed between 0 to 3 and ≥4 involved LNs (hazard ratio = 7.680; 95% confidence interval = 5.051–11.655, $p < 0.0001$). On multivariate analysis, we used the ratio between the number of involved LNs and RLNs. The number of RLNs was found to be a strong independent prognostic factor for NSCLC (hazard ratio = 6.803; 95% confidence interval = 4.137–11.186, $p < 0.0001$).

Conclusion: Complete resection including 10 or more LNs influenced survival at complete NSCLC resection. Four involved LNs seemed to be a benchmark for NSCLC prognosis. The number of involved LNs is a strong independent prognostic factor in NSCLC, and the results of this study may provide new information for determining the *N* category in the next tumor, node, metastasis classification.

*Division of Thoracic Surgery, Department of Surgery, Tokyo Medical University; and †Department of Anatomic Pathology, Tokyo Medical University, Tokyo, Japan.

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Address for correspondence: Hisashi Saji, MD, PhD, Division of General Thoracic Surgery, Department of Surgery, Tokyo Medical University, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. E-mail: saji-q@ya2.so-net.ne.jp

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Key Words: Number of resected lymph nodes, Number of involved lymph nodes, Lymph node dissection, Multivariate analysis.

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Lung cancer has one of the highest worldwide incidence rates and is the leading cause of cancer-related mortality worldwide.[1] In Japan, lung cancer accounts for 60,000 deaths annually, and surgical resections are performed in approximately 27,000 cases, with an overall survival (OS) rate of 60%, according to the annual reports of the Japanese Association for Thoracic Surgery[2] and the Japanese Lung Cancer Registry.[3]

Various pathological and molecular markers have been assessed regarding their status and role in identifying patients at high risk for recurrence. However, the primary tumor, lymph node (LN), and the metastasis (TNM) staging system remain the most important determinant of outcome. Because the prognosis of lung cancer is directly proportional to the presence of LN metastasis, accurate LN assessment is crucial in determining treatment. The role of hilar and mediastinal lymphadenectomy in the staging and treatment of non-small cell lung cancer (NSCLC) remains controversial. Accurate staging of NSCLC requires assessment of the hilar and mediastinal LNs based on pathologic evaluation. In almost all surgical cooperative group trials and clinical settings in Japan, systematic LN dissection in ipsilateral hilar and mediastinal stations is standard. However, there is continual debate regarding the degree to which hilar and mediastinal LNs should be located and removed.

The number of resected LNs (RLNs) has been proven to have prognostic value in colorectal, breast, and bladder cancer.[4–6] Moreover, the number of involved LNs at the time of surgery currently influences staging. However, these items have not yet been incorporated into the latest 7th edition of the TNM classification of lung cancer.[7]

Therefore, we retrospectively investigated the prognostic impact of the number of RLNs and involved LNs in the context of other established clinical prognostic factors, in a series of 928 consecutive patients with NSCLC who underwent complete

resection at Tokyo Medical University. Specifically, we attempted to clarify the number of LNs that should be resected, and the number of involved LNs needed to make an accurate prognosis.

PATIENTS AND METHODS

Patient Selection

From January 2000 to November 2007, a total of 1311 patients underwent resection for primary lung cancer at our institution. Cases of induction therapy, incomplete resection, and limited resection were excluded from this study. Patients whose tumors were classified histologically as small cell lung cancer or low-grade malignant tumors were also excluded. We retrospectively analyzed the remaining 928 consecutive patients with NSCLC who underwent complete resection with curative intent (minimum procedure of lobectomy) with systematic LN dissection of the hilum and mediastinum according to current surgical methods.[8] Patient charts, including pathologic diagnosis and operative reports, were reviewed. Staging was determined according to the international TNM staging system.[9] The histological tumor type was determined according to the World Health Organization classification, 3rd edition. All dissected LNs were examined pathologically and classified according to anatomical location by the numbering system described in the Naruke map.[10] The number of RLNs and involved LNs was confirmed based on the pathological report provided by M.N., J.M., and T.N. These pathologists were blinded to the clinical outcome.

Patient Characteristics

The characteristics of the 928 consecutive patients who underwent surgery for NSCLC were as follows: age, median (range): 65.0 years (22–87 years); sex: 547 (59.0%) men and 381 (41.0%) women; clinical stages: 768 (82.8%) stage I, 84 (9.1%) stage II, and 76 (8.1%) stage III; pathological stage: 677 (72.9%) stage I, 121 (13.0%) stage II, 129 (13.9%) stage III, and 1 (0.2%) stage IV; histopathological diagnosis: 684 (73.7%) adenocarcinomas, 182 (19.6%) squamous cell carcinomas, 52 (5.6%) large cell carcinomas, and 10 (1.1%) others; surgical procedure: 870 (93.8%) lobectomies, 42 (4.5%) bilobectomies, and 16 (1.7) pneumonectomies. The mean number of RLNs was 15 (right side, 15.5; left side, 14.3); the mean number of involved LNs was 4.2 (0–22) (Table 1). The median follow-up time was 3.5 years.

TABLE 1. Patient Characteristics

Variable	Category	*n* (%)
Age (yr)	Mean	65.0
	Range	22–87
Sex	Men	548 (59.0)
	Women	380 (41.0)
Histopathology	Adenocarcinoma	684 (73.7)
	Squamous cell	182 (19.6)
	Large cell	52 (5.6)
	Other	10 (1.1)
Clinical stage	I	768 (82.8)
	II	84 (9.1)
	III	76 (8.1)
Pathological stage	I	677 (72.9)
	II	121 (13.0)
	III	130 (14.1)
Tumor location	Right side	602 (64.9)
	Upper/middle/lower	334/64/204
	Left side	326 (35.1)
	Upper/lower	190/136
Surgical procedure	Lobectomy	870 (93.8)
	Bilobectomy	42 (4.5)
	Pneumonectomy	16 (1.7)
Total number of resected LNs	Mean (range)	15.0 (1–49)
	0–4	59 (6.4)
	5–9	177 (19.1)
	10–14	251 (27.0)
	15–19	201 (21.6)
	≥20	241 (25.9)
Total number of involved LNs in positive cases	Mean (range)	4.2 (1–22)
	0	724 (78.0)
	1–3	122 (13.1)
	≥4	82 (8.9)

LNs, lymph nodes.

Statistical Analysis

We investigated the association between the total number of RLNs or involved LNs and OS. OS was calculated from the date of surgery to the time of death. Observations were censored at final follow-up if the patient was alive. All patients in this series were categorized into four groups according to the number of RLNs less than 5 versus 5 or more, less than 10 versus 10 or more, less than 15 versus 15 or more, and less than 20 versus 20 or more. On analysis of survival differences based on the number of involved LNs, patients were categorized into groups of those with 0 versus 1 or more, less than 3 versus 3 or more, less than 4 versus 4 or more, and less than 5 versus 5 or more of involved LNs. Survival curves were plotted using the Kaplan-Meier method. Differences in survival among the groups were examined using the log-rank test. A two-category comparison was performed using the Pearson χ^2 test and the Student t test for quantitative data. Multivariate analysis was performed using the Cox proportional hazards model to examine any possible association between the ratio of the total number of RLNs and involved LNs and survival, with adjustment for the effects of other potential prognostic factors, including age, sex, histology, tumor factor, and type of surgery performed. All tests were two sided, and p values of less than 0.05 were considered to represent statistically significant differences. StatView version 5.0 software (SAS Institute Inc., Cary, NC) was used for statistical analysis.

Ethical Considerations

The approval of the Institutional Review Board of Tokyo Medical University was obtained, but as this was a retrospective study the need to obtain written informed consent from either the patients or their representatives was waived, in accordance with the AMA Manual of Style (10th edition).

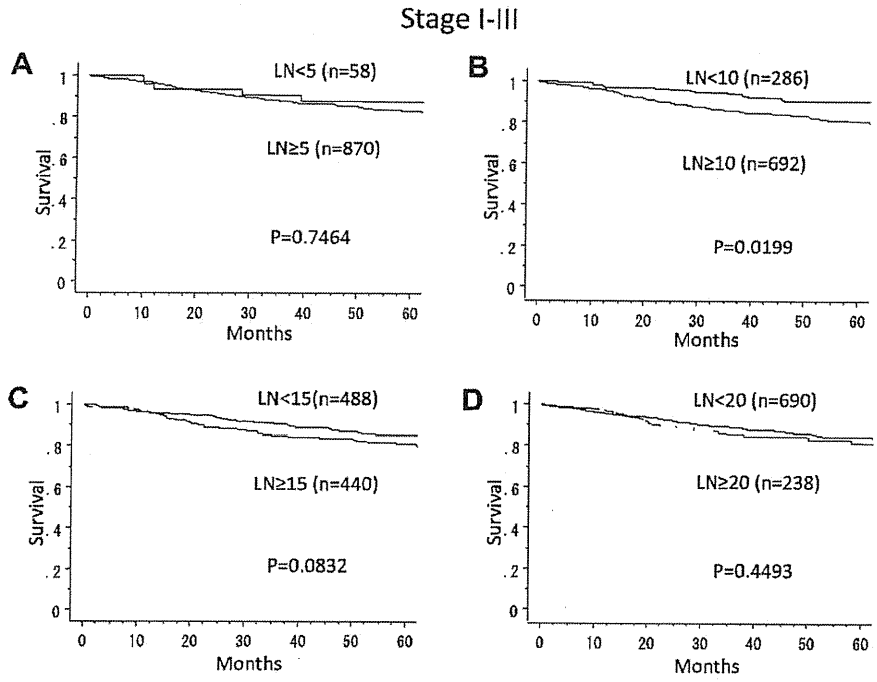

FIGURE 1. Survival curves according to the number of resected LNs at the time of complete resection in patients with stages I to III non-small cell lung cancer. *A*, LN, five nodes as cutoff. *B*, LN, 10 nodes as cutoff; a significant difference in survival was observed. *C*, LN, 15 nodes as cutoff. *D*, LN, 20 nodes as cutoff.

TABLE 2. Correlations between Overall Survival and Total Number of Resected Lymph Nodes

Valuables	*p*	HR	95% CI
5 vs. ≥5	0.7464	1.135	0.528–2.440
6 vs. ≥6	0.5464	1.233	0.624–2.437
7 vs. ≥7	0.1611	1.591	0.831–3.047
8 vs. ≥8	0.744	1.725	0.948–3.140
9 vs. ≥9	0.0217	1.783	1.088–2.923
10 vs. ≥10	0.0199	1.795	1.098–2.912
11 vs. ≥11	0.0295	1.651	1.051–2.595
12 vs. ≥12	0.0473	1.521	1.005–2.302
13 vs. ≥13	0.0907	1.394	0.949–2.050
14 vs. ≥14	0.1137	1.354	0.930–1.973
15 vs. ≥15	0.0832	1.388	0.956–2.014

HR, hazard ratio; CI, confidence interval.

RESULTS

Survival and Number of RLNs

We investigated the prognostic impact of the number of RLNs (mean number of RLNs = 15). Patients were categorized into four representative groups according to the total number of RLNs: less than 5 versus 5 or more, less than 10 versus 10 or more, less than 15 versus 15 or more, and less than 20 versus 20 or more (Figure 1). Table 2 presents each *p* value, hazard ratio (HR), and 95% CI comparing each subgroup categorized according to total number of RLNs. The largest significant difference was found in the total number of RLNs categorized between less than 10 and 10 or more ($p = 0.0199$, HR = 1.795, 95% CI = 1.098–2.912). However, even 15 or more RLNs had no significant prognostic impact on the survival of patients with NSCLC in the present series. There was no sign of incremental improvement in or impairment of survival after the resection and evaluation of 15 or more LNs for curative resection of NSCLC. There were no statistically significant differences in survival according to the total number of RLNs in cases of stage I NSCLC (Figure 2).

As shown in Table 3, the mean numbers of RLNs on both the right and left sides were significantly higher in pN1 or pN2–3 cases than in pN0 cases (right side: $p = 0.0007$, $p = 0.0002$, left side: $p = 0.0068$, $p = 0.0162$, respectively). The mean number of RLNs in cases with right-sided tumors was significantly higher than that in cases with left-sided tumors.

Survival and Number of Involved LNs

We analyzed the number of involved LNs that could provide the most appropriate indicator of OS in NSCLC. Although the incidence of LN involvement was associated with poor prognosis, the largest statistically significant increase in OS was observed between zero to three and four or more involved LNs (HR, 7.680; 95% CI, 5.051–11.655, $p < 0.0001$) (Figure 3). Although patients with no involved LNs had a better outcome than those with 1 to 3 involved LNs, there was no significant difference in survival between the two groups ($p = 0.1831$). Patients with four or more involved LNs had a significantly worse outcome than those with one to three involved LNs ($p < 0.0001$). These results suggest that four or more involved LNs would be the best benchmark of OS in NSCLC (Figure 4).

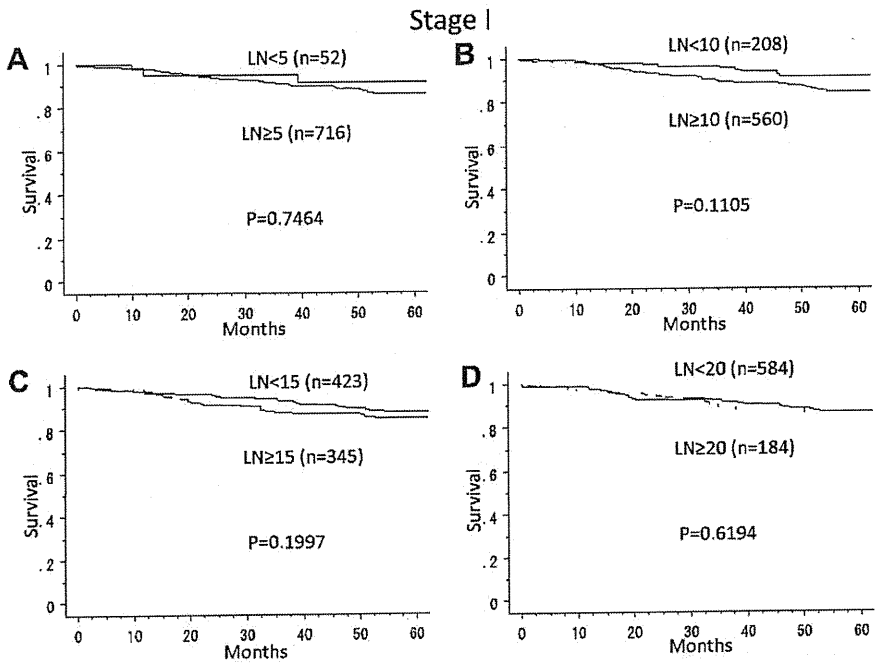

FIGURE 2. Survival curves according to the number of resected LNs at the time of complete resection in patients with stage I, non-small cell lung cancer. *A*, LN, five nodes as cutoff. *B*, LN, 10 nodes as cutoff. *C*, LN, 15 nodes as cutoff. *D*, LN, 20 nodes as cutoff. No significant difference in survival was observed in any group.

TABLE 3. Mean Number of Resected Lymph Nodes on Right or Left Side

	Mean Number	*p*
Right side lymph nodes, $n = 602$		
Total	15.5	
pN0	14.7	
pN1	18.2	0 vs. 1: $p = 0.0007$[a]
pN2–3	19.0	0 vs. 2–3: $p = 0.0002$[a]; 1 vs. 2–3: $p = 0.7199$
Left side lymph nodes, $n = 326$		
Total	14.3	
pN0	13.5	
pN1	16.6	0 vs. 1: $p = 0.0068$[a]
pN2–3	16.3	0 vs. 2–3: $p = 0.0162$; 1 vs. 2–3: $p = 0.8985$
Right vs. left		$p = 0.0323$[a]

[a] Statistical significance.

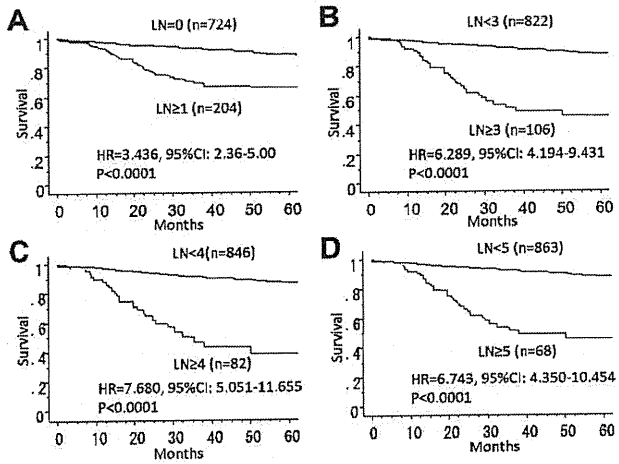

FIGURE 3. Survival curves according to the number of involved LNs at the time of complete resection in patients with stages I to III, non-small cell lung cancer. *A*, LN, one node as cutoff. *B*, LN, three nodes as cutoff. *C*, LN, four nodes as cutoff. *D*, LN, five nodes as cutoff. Although the incidence of lymph node involvement was statistically associated with poor prognosis, the largest statistically significant increase in OS was seen between zero to three and 4 or more involved LNs.

Correlations between Number of RLNs, Involved LNs and pN Status

Before analyzing the possibility of RLNs and involved LNs as possible independent prognostic factors by multivariate analysis, we examined whether RLNs, involved LNs and pN status were confounding factors. The mean and range of the total number of RLNs in our series were 15.0 and 1 to 49, respectively. The mean number of RLNs was significantly increased in pN1 or pN2–3 cases compared with pN0 cases ($p < 0.0001$ and $p < 0.0001$, respectively), whereas the mean and range of the total number of involved LNs in our pN-positive series were 4.2 and 1 to 22, respectively. The mean numbers of involved LNs in pN1 and pN2–3 cases were 2.15 and 6.56, respectively. The number of involved LNs was significantly higher in pN2–3 cases than in pN1 cases ($p < 0.0001$). These results demonstrate that each of these prognostic factors (i.e., the number of RLNs and involved LNs,

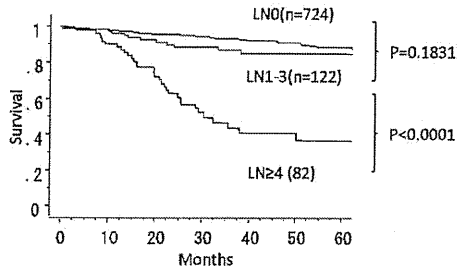

FIGURE 4. Survival curves according to the number of involved LNs at the time of complete resection in patients with stages I to III, non-small cell lung cancer. Although patients with no involved LNs had a better prognosis than those with one to three involved LNs, there was no significant difference in survival between the two groups. Patients with four or more involved LNs had a significantly worse outcome than those with one to three involved LNs.

and pN status) were confounding factors in our series. Therefore, in the subsequent multivariate survival analysis, we used the ratio between the number of involved and RLNs to reflect both factors in the multivariate analysis similarly to a previously reported method.[11]

Multivariate Survival Analysis

We performed multivariate analysis to confirm the prognostic impact of the total number of RLNs and involved LNs in NSCLC, using the ratio between the number of involved and RLNs, according to a previously observed correlation.[11] As shown in Table 4, RLNs strongly correlated with poor prognosis on multivariate analysis after adjustments for sex, age, histology, tumor factor, and surgical procedure. We therefore concluded that RLN was a strong independent prognostic factor for NSCLC (HR, 6.803; 95% CI, 4.137–11.186, $p < 0.0001$). Other independent prognostic factors identified on multivariate analysis included sex (HR, 0.620; 95% CI 0.401–0.958, $p = 0.0313$), age (HR, 1.598, 95% CI 1.090–2.341, $p = 0.0162$), and T factor (HR, 0.392, 95% CI, 0.256–0.600, $p < 0.0001$).

DISCUSSION

We set out to determine the number of LNs that should be resected, and the number of involved LNs for the accurate prediction of outcome in resectable cases of lung cancer. Opinions still vary among surgeons as to whether to remove all, some, or none of the mediastinal LNs at the time of pulmonary resection for lung cancer, and practices vary worldwide. In almost all surgical cooperative group trials in North America, LN sampling is standard, whereas systematic LN dissection is standard in Japan.

LN status is a major determinant of stage and survival in patients with lung cancer. However, the role of mediastinal lymphadenectomy in the staging and treatment of NSCLC remains controversial. The present results indicate that patient survival after complete NSCLC resection is associated with the number of LNs harvested during surgery. The largest significant difference was observed in the total number of RLNs categorized between less than 10 and 10 or more ($p = 0.0199$, HR = 1.795, 95% CI = 1.098–2.912). Patients with 10 or more RLNs had significantly worse outcomes than those with less than 10 RLNs (Figure 1), contrary to the findings of previous studies of stage I NSCLC cases.[11–13] As shown in Table 3, the mean number of RLNs on both the right and left sides was significantly higher in pN1 or pN2–3 cases than in pN0 cases (right side: $p = 0.0007$, $p = 0.0002$; left side: $p = 0.0068$, $p = 0.0162$, respectively), which may be one reason why patients with NSCLC with 10 or more RLNs had a worse outcome than those with less than 10 RLNs. According to the results of the American College of Surgeons Oncology Group (ACOSOG) Z0030 study, a higher N stage was also associated with increased LN removal (N0: 19.2 ± 10.1; N1: 22.8 ± 10.9; N2: 24.5 ± 10.8; $p = 0.043$).[12] This is possibly because surgeons tend to harvest more LNs in patients with LN-positive disease at the time of surgery, in expectation of therapeutic benefit. However, even 15 or more

TABLE 4. Univariate and Multivariate Survival Analyses

Variable	Category	*n*	Univariate Analysis *p*	Multivariate Analysis HR	95% CI	*p*
Sex	Men	548	0.011[a]	0.620	0.401–0.958	0.0313[a]
	Women	381				
Age (yr)	<70	690	0.0209[a]	1.598	1.090–2.341	0.0162[a]
	≥70	338				
Histopathology	Non-adenocarcinoma	244	0.015[a]	0.790	0.518–1.203	0.2719
	Adenocarcinoma	684				
T factor	T2–3	433	<0.0001[a]	0.392	0.256–0.600	<0.0001[a]
	T1	495				
Surgical procedure	Lobectomy	912	0.0136[a]	2.521	0.768–8.280	0.1273
	Pneumonectomy	16				
RLNs	<0.4	882	<0.0001[a]	6.803	4.137–11.186	<0.0001[a]
	≥0.4	46				

[a] Statistical significance.
RLNs, ratio between the number of involved and resected lymph nodes; CI, confidence interval; HR, hazard ratio.

RLNs had no significant impact on OS in patients with NSCLC in the present series, contrary to the results of a previous large study.[13] There appeared to be neither incremental improvement nor impairment of survival after resecting and evaluating 15 or more LNs with curative intent in NSCLC in the current series. One possible explanation for this is that the presence of approximately 10 dissected LNs increases the staging accuracy.

There was no significant difference in survival according to the total number of RLNs in stage I NSCLC in the current series. Recent retrospective studies from cancer registries,[14] nonrandomized trials,[15] and other institutions,[16–21] have indicated that the number of RLNs is associated with better OS.[16–19] Although LN removal may be therapeutic, the therapeutic benefit is likely to be small for patients with stage IA NSCLC, because all LNs in stage IA should be negative. The other, less likely explanation, is that a more extensive LN dissection such as systematic mediastinal LN dissection may be therapeutic, at least in stage I NCSLC.

The present analysis shows that an increasing number of RLNs during complete NSCLC resection is associated with a statistically significant difference in survival, which peaks at 10 to 14 LNs. Some studies have recommended that the minimum requirements for accurate nodal staging must include the removal of at least six LNs from hilar and mediastinal stations.[7,22,23] However, others have recommended the examination of a minimum of 10 LNs and at least three LN stations.[14,19] Although we are reluctant to recommend a definitive optimal number of LNs, the current data support the conclusion that an evaluation of nodal status should include at least 10 LNs.

Nodal involvement is the most important prognostic factor in determining survival for many malignant tumors. These factors are represented by the N category in the TNM classification and are grouped according to the anatomical location and/or number of LN involvement. In the most recently published 7th edition of the *TNM Classification of Malignant Tumors* (2009),[24] the number of involved LNs is included in the definition of pN factors in breast, stomach, esophageal, and colorectal cancer, and pN status shows a significant correlation with outcome. The nodal system in this edition in lung cancer is still based on the anatomical location of involved LNs. The Naruke map and the American Thoracic Society map have been combined into the International Association for the Study of Lung Cancer map, and the definition of the border between N1 and N2 has been changed, because of its complexity and ambiguity. However, this change is based on the anatomical location, not on the biological issue. In the current study, we predicted patient outcome after complete NSCLC resection according to the number of involved and RLNs, as previous reports have suggested.[25,26] Recently, Asamura and coworkers[27] have provocatively suggested that the number of metastatic LNs provides more accurate pathologic nodal staging than the current method of considering anatomical location of involved nodes. The largest statistically significant increase in OS was observed between zero to three and four or more involved LNs (HR, 7.680; 95% CI, 5.051–11.655; $p < 0.0001$) (Figure 2). Therefore, the current data indicate that four or more involved LNs serve as a good indicator of outcome after complete NSCLC resection. Because it is possible that the number of RLNs and involved LNs may indicate the quality of surgery in the determination of accurate staging and survival impact after complete NSCLC resection, we used RLNs as a prognostic predictor on multivariate analysis. In addition to T stage, RLNs had a strong independent effect on survival in patients with complete NSCLC resection in the present study. Indeed, the 5-year survival ratio of patients with RLNs ≥ 4 is similar to that of patients with pN2 disease in our series (data not shown). Although the nodal classification according to the number of involved LNs is simple and easy to be incorporated in the next TNM classification, there are a few limitations that are not helpful in deciding treatment preoperatively because it is mainly based on pathological assessment. However, this may change in the future with the development of new imaging device.

Our data suggested that the number of involved LNs expands pN category information and may provide additional information for the pN category of the next TNM classification. Further large-scale cohort studies, including global prospective validation analyses and multi-institutional studies are warranted.

CONCLUSION

We retrospectively evaluated the prognostic impact of the number of RLNs and involved LNs on the survival of patients with complete NSCLC resection. We found that 10 or more LNs harvested with complete LN dissection possibly influenced survival after complete NSCLC resection. Moreover, the presence of four involved LNs seemed to be a good indicator of outcome after complete NSCLC resection. The number of involved LNs was a strong independent prognostic factor in NSLC, and this may provide new information for the N categorization of the next TNM classification.

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Klotho predicts good clinical outcome in patients with limited-disease small cell lung cancer who received surgery

Jitsuo Usuda[a,*], Shuji Ichinose[a], Taichirou Ishizumi[a], Keishi Ohtani[a], Tatsuya Inoue[a], Hisashi Saji[a], Masatoshi Kakihana[a], Naohiro Kajiwara[a], Osamu Uchida[a], Masaharu Nomura[b], Tatsuo Ohira[a], Norihiko Ikeda[a]

[a] Division of Thoracic Surgery, Department of Surgery 1, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[b] Department of Pathology, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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MRP1

ABSTRACT

Background: The important role of surgery in early-stage small cell lung cancer (SCLC) has been recognized, and curative surgical resection is recommended. However, the role of adjuvant chemotherapy for stage I SCLC has not yet been evaluated, and novel approaches focusing on the specific genomic characteristics of SCLC may be invaluable for customized therapy. In this study, we focused on the *Klotho* gene, which is an anti-aging gene known to be a potential tumor suppressor. We investigated whether the expression of Klotho, assessed by immunohistochemistry, can predict survival in patients with resected SCLC.

Methods: The medical records of patients diagnosed as having limited-disease (LD) SCLC and treated by surgical resection ($n=30$) at Tokyo Medical University Hospital were retrospectively reviewed. The expression status of Klotho, and of the ATP-binding cassette (ABC) transporters MRP1, MDR and breast cancer resistant protein (BCRP), which can cause resistance to anticancer drugs, including irinotecan, was assessed by immunohistochemical analysis in resected surgical specimens of patients with early-stage SCLC.

Results: Of the 30 patients, Klotho expression was seen in the specimens from 18 patients (60.0%), but not in those of the remaining 12 patients (40.0%). The immunostaining for Klotho was mostly localized in the cytoplasm. The expression of Klotho was significantly associated with the overall survival (OS) (ratio 0.088; 95% confidence interval 0.019–0.409; $P=0.002$). The administration of perioperative chemotherapy had no significant effect in improving the survival, as assessed by the Kaplan–Meier method. However, the patients showing Klotho expression in the resected specimens in p-stage I and II, may have benefited from perioperative chemotherapy. A multivariate analysis revealed no significant association between the expression status of MRP1, MDR or BCRP and the OS.

Conclusion: Expression of Klotho was predictive of a favorable outcome following resection in limited-disease SCLC patients, and the Klotho expression status may serve as a new biomarker for the need of additional therapies to be developed in the future.

1. Introduction

Small cell lung cancer (SCLC) accounts for approximately 15% of all new cases of lung cancer [1,2]. Most patients with SCLC present with advanced nodal disease, with or without systemic involvement, and SCLC accounts for less than 5% of the cases in large surgical series [3]. SCLC is considered as a chemosensitive disease, and the reported initial response rates to platinum-based chemotherapy are equal to or more than ≥60%, and the complete response rates, 20–30% [3–6]. However, the reported median survival time and 2-year survival rate range from 7 to 10 months and 10–20%, respectively. This discrepancy can possibly be explained by the biological behavior of SCLC, which is associated with early dissemination to the regional lymph node and/or distant metastasis in more than 90% of patients. Even patients with early-stage SCLC probably have micro-metastatic deposits at distant sites, that carry the potential for proliferation [7]. It is recognized that the clinical staging is not correlated with the pathologic staging in cases of SCLC, with the reported concordance between clinical and pathologic TNM staging being only 58% [7].

* Corresponding author at: Department of Thoracic Surgery, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
Tel.: +81 3 3342 6111; fax: +81 3 3349 0326.
E-mail address: jusuda@tokyo-med.ac.jp (J. Usuda).

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The role of surgery in early-stage SCLC has been reviewed, and the ACCP (American College of Chest Physician) evidence-based clinical practice guideline recommends surgical resection with curative intent after invasive mediastinal staging and extrathoracic imaging (head MRI, abdominal CT plus bone scintigraphy) in patients with stage I SCLC [8]. However, the role of adjuvant chemotherapy has not yet been evaluated in prospective randomized trials [9–11]. In order to improve the efficacy of the treatment for SCLC, novel approaches focusing on the specific genomic characteristics of individual tumors may be invaluable. Therefore, identification of predictive and/or prognostic biomarkers is essential for customized therapy in SCLC patients.

Recently, Chiappori et al. reported ribonucleotide reductase 1 (RRM1) and topoisomerase 2α (Topo2α) expression as predictive biomarkers of chemotherapeutic efficacy in SCLC [12]. While no significant associations was found between either response or progression-free survival, and the presence/absence of the expressions of ATP-binding cassette (ABC) transporters such as MRP1, MDR, and MRP2, in patients with SCLC, expression of the breast cancer resistant protein (BCRP), which is one of the ABC transporters reported to be associated with resistance to anticancer drugs, including irinotecan, mitoxantrone and doxorubicin, was found to be a significant predictor of both the response and progression-free survival in SCLC patients receiving chemotherapy [13–16].

The Klotho gene, which is a 1014-amino acid single-pass transmembrane protein, has been characterized as a systemic anti-aging hormone and was originally identified in mice homozygous for the mutated allele ($kl^{-/-}$) [17–19]. These mice show a human-like aging-related syndrome and develop multiple disorders such as hypogonadism, ectopic calcification, osteoporosis, skin atrophy, and pulmonary emphysema [18]. In contrast, transgenic mice overexpressing Klotho show an extended life span that is 30% longer in males and 20% longer in females. Wolf et al. reported that Klotho is a potential tumor suppressor in breast cancer [20] and Lee et al. reported that Klotho encoding the secreted Wnt antagonist that acts as a tumor suppressor may be a candidate for epigenetic silencing in human cervical carcinoma [21]. Recently, we demonstrated the significant prognostic value of the Klotho expression status in patients with LCNEC, and demonstrated that and positive immunostaining for Klotho represented a novel biomarker of a favorable outcome in these patients [22]. Therefore, we hypothesized that the Klotho gene may play an important role in regulating cell growth and that Klotho expression may also serve as a new biomarker for the treatment outcome in patients with SCLC. In this study, we conducted a retrospective investigation to determine whether the immunohistochemical expression status of Klotho and of ABC transporters, such as BCRP, can predict the survival following resection in SCLC patients.

2. Materials and methods

2.1. Patient selection

Of 1722 patients who underwent surgical resection for primary lung cancer between January 1994 and December 2005 at Tokyo Medical University Hospital, 30 with the histological diagnosis of SCLC in the stage of limited disease (LD) were enrolled as the subjects of this study. All the histological diagnoses were made by experienced pathologists at the Department of Pathology, Tokyo Medical University Hospital. This retrospective study was conducted with the approval of the Ethical Committee of Tokyo Medical University.

2.2. Immunostaining for Klotho

Immunohistochemical staining for Klotho was performed on 4 μM-thick formalin-fixed, paraffin-embedded tissue sections [20–22]. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 10 min. All the slides were heated to 95 °C by exposure to microwave irradiation for 20 min. The slides were then cooled for 1 h at room temperature and washed in phosphate buffer solution (PBS). Non-specific binding was blocked by pre-incubation with 1% BSA for 30 min. After washing with PBS, the slides were incubated for 1 h at room temperature with anti-MDR antibody (JSB-1; Invitorgen, Camarillo, CA, USA), or anti-MRP-1 antibody (MRPm6; Sanbio, Uden, Netherlands), anti-BCRP antibody (Bxp-21; Chemicon, Temecula, CA, USA), or anti-Klotho antibody, KM2076 (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan). Antibody staining was considered positive when at least 10% of the tumor cells were stained, based on the use of the 10% cutoff level in several previous studies [23]. All the slides were examined by two observers who had no knowledge of the patients' clinical data.

2.3. Statistics

Clinical information was extracted from the medical records. The disease stage was expressed according to the TNM classification based on the International Union Against Cancer (UICC) staging system. Statistical analyses were performed using SPSS for Windows. The Kaplan–Meier method was used to determine the patients' survival [23–26]. Overall survival was defined as the time (in months) from the date of surgery to the date of death or the date patients were last known to be alive at October 2010. We assessed the univariate effect of each variable on the survival using the log-rank test for the categorical predictors, and the univariate Cox model for the continuous predictors [23–26]. The expression statuses of MRP1, MDR and Klotho, presence/absence of lymph node metastasis, and presence/absence of lymphangioinvasion were candidates with 0.05 significance level for entry into the model. Then, the survival curves of the different patient categories were compared.

3. Results

3.1. Patient characteristics

The clinicopathological characteristics of the patients are listed in Table 1. Their mean age at the time of surgery was 61.9 years (range, 46–77). Of the 30 patients, 25 were men, and 5 were women. The surgical procedures performed included lobectomy in 28 patients and pneumonectomy in 2 patients. The distribution of the clinical stages in the patients was stage IA in 11 patients, stage IB in 6 patients, stage IIA in 2 patients, stage IIB in 3 patients, stage IIIA in 6 patients, and stage IIIB in 2 patients. It has long been recognized that in patients with SCLC, the clinical staging is not well-correlated with the pathologic staging, and the IASLC staging project reported that the concordance between clinical and pathologic TNM staging was only 58% [1–4]. In our study, the distribution of the pathological stages in the 30 patients of SCLC who had undergone surgical resection was as follows: stage IA in 8 patients, stage IB in 7 patients, stage II A in 4 patients, stage IIB in 3 patients, stage IIIA in 3 patients, stage IIIB in 4 patients, and stage IV in 1 patient. In Table 1, we showed the number of p-stage, and 4 cases were p-stage IIIB, and 1 case was p-stage IV. The four cases with p-IIIB were T4, due to pulmonary metastasis in the same lobe as the primary lesion, and the one case with p-IV was M1, due to pulmonary metastasis in the different lobe on the same side. The number of 5 cases in c-IA and 5 cases in c-IB accorded with the number of p-IA and p-IB. Three

Table 1
Clinicopathological characteristics of 30 patients with SCLC.

Patient characteristics	No. of cases
No. of patients	30
Age (mean)	46–77 (61.9)
Gender	
Male	25
Female	5
Surgical procedure	
Lobectomy	28
Pneumoncetomy	2
c-Stage (p-stage)	
IA	11 (8)
IB	6 (7)
IIA	2 (4)
IIB	3 (3)
IIIA	6 (3)
IIIB	2 (4)
IV	0 (1)
Chemotherapy	
Yes	19
No	11
Alive	
Yes	18
No	12

cases in c-IIA were turned out to be in p-IA, and 2 cases in c-IIB were turned out to be in p-IB. The concordance rate between the clinical and pathologic staging was 46.7% overall (14/30), but slightly higher for stage I SCLC, being 58.8% (10/17). During the study period, 19 patients underwent chemotherapy before or after the surgery, 12 patients died (40.0%), and 18 patients (60.0%) survived for more than 60 months (Table 1). Eight patients underwent chemotherapy before the surgery, and the regimens were cisplatin plus etoposide in 5 patients and carbplatin plus etoposide in 3 patients. They received 2 courses of the chemotherapy. Eleven patients underwent chemotherapy after the surgery, and the regimens were cisplatin plus etoposide in 3, carboplatin plus etoposide in 6, and carboplatin plus irinotecan in 2. They received 2–4 courses after the surgery.

3.2. Expression status of the ABC transporter and anti-aging protein Klotho proteins in SCLC

Recently, the ABC transporters have been reported as having pivotal roles in the mechanisms of development of drug resistance, and that BCRP may represent an ideal molecular target for the treatment of SCLC [13–15]. Therefore, in order to elucidate the association between the expression status of the ABC transporters, such as BCRP, and the survival prognosis following surgical resection in patients with SCLC, we performed immunohistochemistry for the ABC transporters. For the ABC transporter proteins BCRP, MRP1 and MDR, both membranous and cytoplasmic immunostainings were recognized, as shown in Fig. 1. All 30 (100%) of the 30 patients with SCLC were BCRP-positive, 11 (33.3%) were MRP1-positive, and 8 (26.7%) were MDR1-positive (Table 2). A multivariate analysis revealed that the expression statuses of the ABC transporter proteins BCRP, MRP1 and MDR1 were not significantly associated with the overall survival (Table 2).

3.3. Association between Klotho expression and the overall survival

In order to clarify whether the expression of Klotho may serve as a predictor of survival following surgical resection in patients with SCLC, and whether Klotho can serve as a new biomarker to determine the need for additional therapies such as postoperative chemotherapy, we conducted immunohistochemical analysis of Klotho expression. Klotho expression was associated with cytoplasmic immunostaining (Fig. 2), as reported previously [20–22], and 18 (60%) of the 30 patients with SCLC were Klotho(+) (Table 2). Fig. 3 shows the Kaplan–Meier survival curves for the Klotho-positive (18 patients) and Klotho-negative patients (12 patients); a comparison of the two revealed that the expression of Klotho was significantly associated with the survival following resection in the SCLC patients ($P=0.002$). Moreover, Klotho expression was identified as a good prognostic factor by multivariate analysis (hazard ratio = 0.088; 95% confidence interval: 0.019–0.409; $P=0.002$) (Table 2).

Table 2
Prognostic significance on overall survival (multivariate analysis).

	Cases	HR (95% CI)	*P* value[a]
Klotho expression			
Negative	12	1	
Positive	18	0.088 (0.019–0.409)	0.002
BCRP			
Negative	0		
Positive	30		
MRP1			
Negative	19	1	
Positive	11	1.207 (0.263–5.532)	0.809
MDR-1			
Negative	22	1	
Positive	8	1.805 (0.269–12.10)	0.543

[a] Tested in Cox regression model with Klotho expression, lymph node, lymphangioinvasion, chemotherapy, HR, hazard ratio; CI, confidence interval.

Table 3 shows the clinical features of the patients with SCLC showing Klotho expression. Of the 18 patients with Klotho(+) SCLC, 7 were p-IA, 5 were p-IB, 3 were p-IIA, 2 were p-IIB and 1 was p-IIIB. Sixteen patients were alive and 2 died of SCLC (Table 3). Five of the 6 Klotho(+) patients with a positive lymph node status, including p-IIA, p-IIB and p-IIIB, were alive, and 11 of the 12 with a negative lymph node status, including p-IA and p-IB, were also alive (Table 3). Eight of the 18 Klotho(+) SCLC patients received chemotherapy before or after the surgery, and the remaining 10 did not receive chemotherapy (Table 3). There were 3 patients with Klotho expression (+) in p-stage IIA and 1 patients with Klotho expression in p-stage IIB who received chemotherapy (Table 3). From the results, they may have benefited from perioperative chemotherapy.

Of all the 30 patients, irrespective of the Klotho expression status, 19 received perioperative chemotherapy while the remaining 11 did not. Fig. 4 shows the Kaplan–Meier survival curves for the 19 patients who received perioperative chemotherapy and the 11 patients who did not receive perioperative chemotherapy; the results revealed that administration of perioperative chemotherapy had no significant influence on the survival following resection in the SCLC patients ($P=0.407$).

Moreover, multivariate analysis did not identify perioperative chemotherapy as a significant prognostic factor (hazard ratio = 0.67; 95% confidence interval: 0.12–3.71; $P=0.648$) (Table 4).

Table 3
Pathological stage for patients with SCLC expressing Klotho.

p-Stage	No. of cases (chemo)	Alive (cases)
IA	7 (2)	6
IB	5 (2)	5
IIA	3 (3)	3
IIB	2 (1)	1
IIIA	0 (0)	0
IIIB	0 (1)	1
IV	0 (0)	0
Total	18 (8)	16

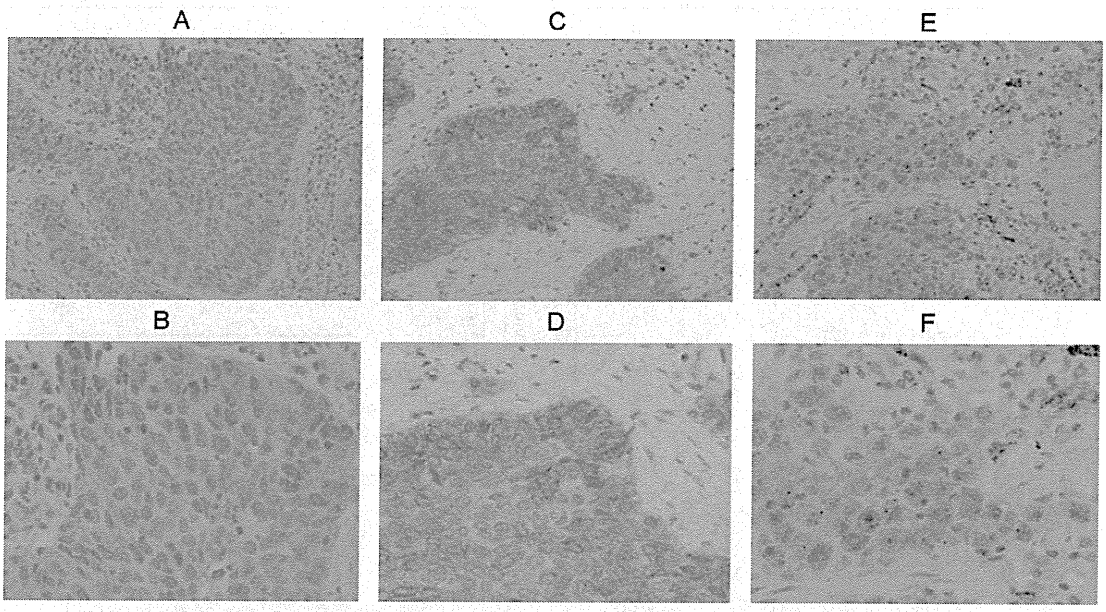

Fig. 1. Immunohistochemical staining of resected specimens of small cell lung cancer with anti-BCRP antibody (A, 100×; B, 400×), anti-MRP1 antibody (C, 100×; D, 400×), and anti-MDR (E, 100×; F, 400×).

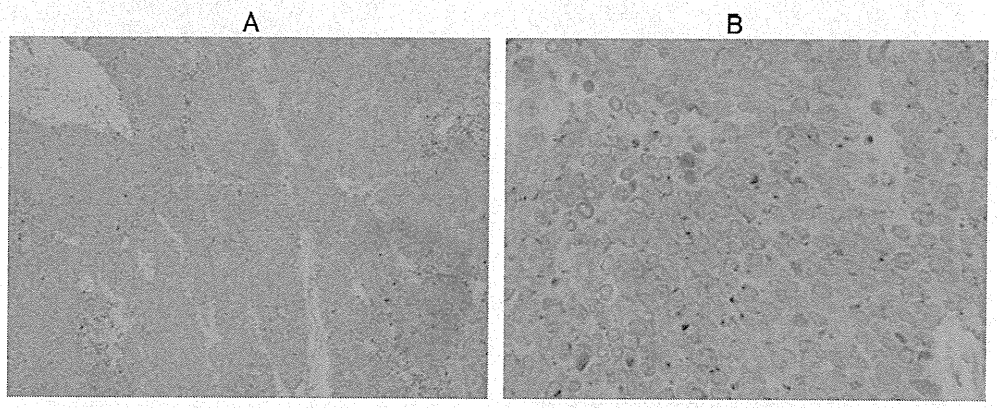

Fig. 2. Immunohistochemical staining of resected specimens of small cell lung cancer with anti-Klotho antibody (KM2076) (A, 100×; B, 400×).

Table 4
Prognostic significance on overall survival (multivariate analysis).

	Cases	HR (95% CI)	*P* value[a]
Lymph node			
Meta (−)	14	1	
Meta (+)	16	2.28 (0.68–7.65)	0.168
Lymphangioinvasion			
Absent	15	1	
Present	15	1.03 (0.33–3.21)	0.960
Chemotherapy			
Yes	19	1	
No	11	0.67 (0.12–3.71)	0.648

[a] Tested in Cox regression model with Klotho expression, lymph node, lymphangioinvasion, chemotherapy. HR, hazard ratio; CI, confidence interval.

3.4. Association between the presence/absence of lymph node metastasis, lymphangioinvasion and survival

Both the presence of lymph node metastasis and that of lymphovascular invasion have been suggested as potential markers of a poor outcome after surgery among SCLC patients, however, the results have been controversial and inconsistent [1–5]. In this study, however, no significant difference in the overall survival was observed between patients with (n = 16) and without lymph node metastasis (n = 14), and between those with (n = 15) and without lymphangioinvasion (n = 15) in the primary SCLC tumor, as shown in Figs. 5 and 6. Multivariate analysis did not identify either lymph node metastasis (hazard ratio = 2.28; 95% confidence interval: 0.68–7.65; P = 0.169) or lymphangioinvasion (hazard ratio = 1.03; 95% confidence interval: 0.33–3.21; P = 0.960) as a significant prognostic factor (Table 4).

4. Discussion

SCLC is characterized by early dissemination, and 70–80% of patients have widely metastatic disease at diagnosis [1–3]. SCLC is considered as a chemosensitive disease, however, despite initial response rates of equal to or more than 60% and complete response rates of 20–30%, the reported median survival time and 2-year survival rate range from 7 to 10 months and 10–20%, respectively [1–3,7,8]. Therefore, for obtaining improvements in the survival, identification of suitable biomarkers is necessary for defining the subset of patients with potentially poor outcomes, so that these patients can be targeted for additional therapies, especially after surgical resection for stage I SCLC [8,9,12]. Identification

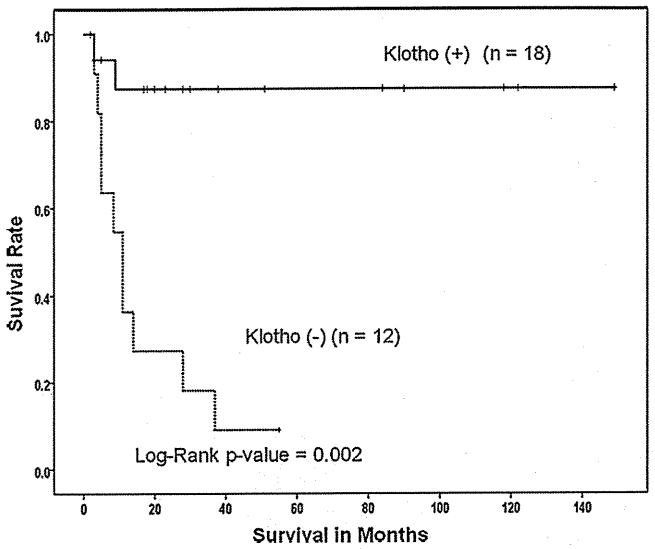

Fig. 3. Kaplan–Meier survival plot showing the survival times of the 30 patients with SCLC whose resected specimens showed positive staining for Klotho ($n=18$), negative ($n=12$) staining for Klotho. The Klotho(+) SCLC patients showed significantly better survival than the Klotho(−) SCLC patients ($P=0.002$).

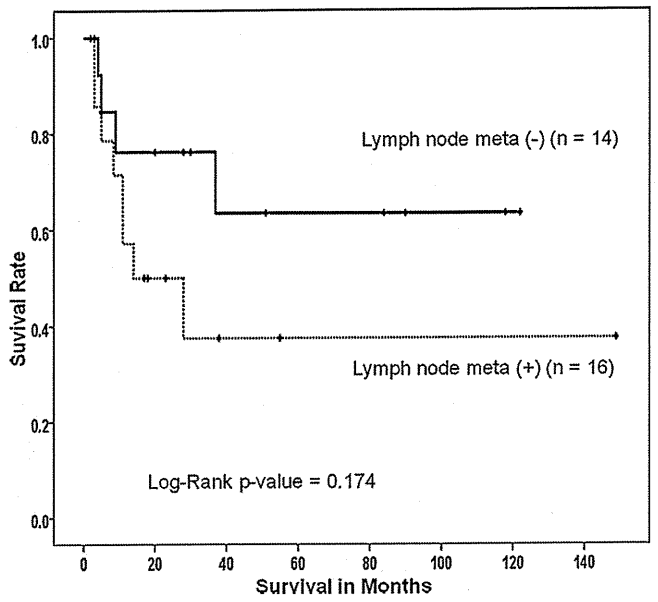

Fig. 5. Kaplan–Meier survival plot showing the survival curves of the SCLC patients with lymph node metastasis ($n=16$), without lymph node metastasis ($n=14$). The lymph node metastasis status had no significant effect on the survival ($P=0.174$).

of predictive and prognostic molecular markers is necessary for individualized treatment. In this retrospective study, we demonstrated the significant prognostic value of the Klotho expression status in SCLC, and demonstrated that Klotho expression may play an important role in predicting the outcome in patients with SCLC. Wolf et al. reported that Klotho, which has been reported as a potent tumor suppressor gene in breast cancer, may serve as a predictor of the breast cancer risk among subjects with BRCA 1 mutations [21]. They also reported that Klotho overexpression specifically reduced the colony formation of breast cancer cells [21]. Recently, we reported, based on an analysis of patients with resected large cell neuroendocrine carcinoma (LCNEC), that the expression of Klotho, demonstrated by immunostaining, served as a new biomarker for a favorable outcome [22]. The rate of Klotho expression in LCNEC (33.3%) was lower as compared with that in SCLC (60.0%) [22]. In addition, Klotho was not found to be a key biomarker for a definitive diagnosis of neuroendocrine tumors, including LCNEC and SCLC.

For successful treatment of SCLC, drug resistance is considered as a major obstacle, and Kim et al. reported that expression of BCRP, demonstrated by immunohistochemistry, was significantly associated with the response and progression-free-survival in SCLC patients, and that BCRP may be an ideal therapeutic target in SCLC patients [13]. However, in this study, multivariate analysis did not identify expressions of the ABC transporters, including BCRP, MRP1 and MDR, as prognostic factors (Table 2). In particular, expression of BCRP was noted in all 30 patients (100%), all of whom were heavy smokers. There was no significant association between the expression of BCRP and that of Klotho.

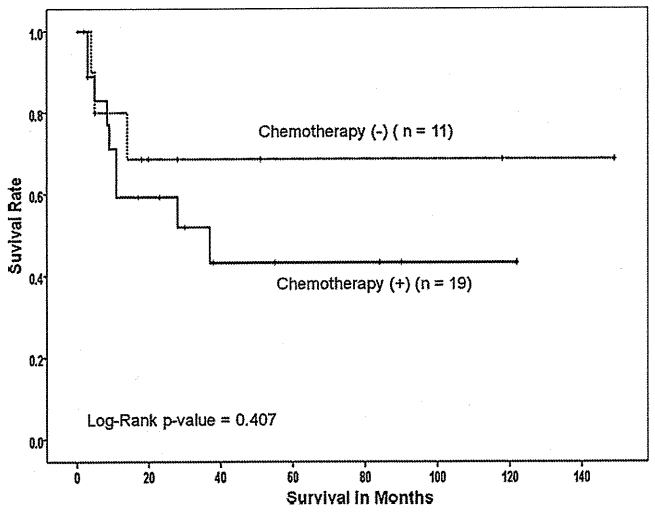

Fig. 4. Kaplan–Meier survival plot showing the survivals of the SCLC patients administered chemotherapy ($n=11$), not administered chemotherapy ($n=19$). Chemotherapy had no significant effect on the survival ($P=0.407$).

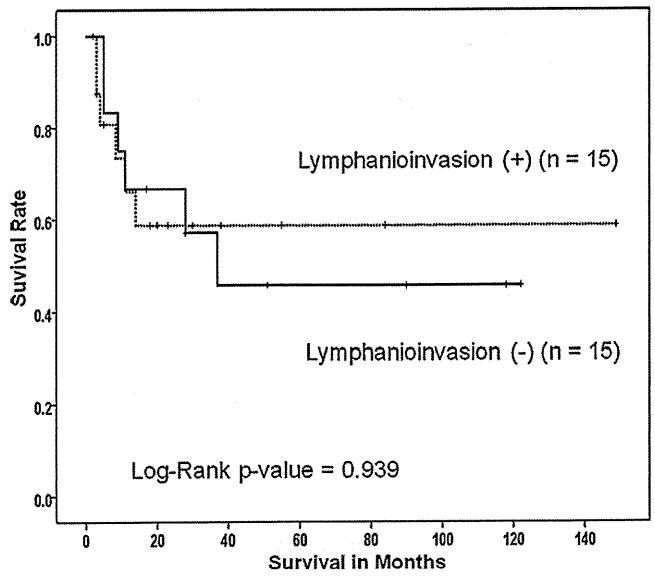

Fig. 6. Kaplan–Meier survival plot showing the survival curves of the SCLC patients with lymphangioinvasion (+) ($n=15$), lymphangioinvasion (−) ($n=15$). The lymphangioinvasion status had no significant effect on the survival ($P=0.939$).

Recently, Yu et al. reported their results of analysis of the outcomes of SCLC patients in the National Cancer Institute Surveillance Epidemiology and End Results (SEER) database treated from 1988 to 2004 [8]. They concluded that surgery seemed to offer reasonable OS outcomes in a cohort of stage I patients who underwent lobectomy [8]. Even though the role of adjuvant chemotherapy has not been evaluated in prospective randomized trials, there are several reports that suggest a survival benefit of adjuvant chemotherapy in stage I SCLC patients. The National Comprehensive Cancer Network guidelines have recommended lobectomy and mediastinal lymph node dissection, followed by chemotherapy, as the preferred treatment in patients with clinical stage I SCLC [8,9]. However, in this study, as shown in Fig. 4, the survival rate of the patients who did not receive chemotherapy was slightly higher than that of the patients who did receive chemotherapy although the difference was not significant, and multivariate analysis excluded perioperative chemotherapy as a prognostic factor (Table 4, Fig. 4). Because in Fig. 4 10 of the 11 patients who did not receive chemotherapy were Klotho(+) SCLC patients, in order to improve the survival of patients with SCLC we hypothesized that for Klotho(−) SCLC patients, further treatment, including chemotherapy, may offer benefit.

The number of patients with SCLC diagnosed in the early stage is rather limited, therefore, prospective randomized trials are very difficult to conduct. ACCP evidence-based clinical practice guidelines recommend (level 2C recommendation) platinum-based adjuvant chemotherapy for stage I SCLC patients who have undergone surgical resection with curative intent [3]. The survival benefit offered by perioperative chemotherapy in Klotho(+) early-stage SCLC patients should be evaluated further in large multi-institutional trials through international multidisciplinary collaboration.

In summary, we demonstrated that the expression status of Klotho was a significant determinant of the prognosis following resection in SCLC patients. In the future, evaluation of the expression status of this marker may enable customized treatment of patients with this aggressive histotype of lung cancer.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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