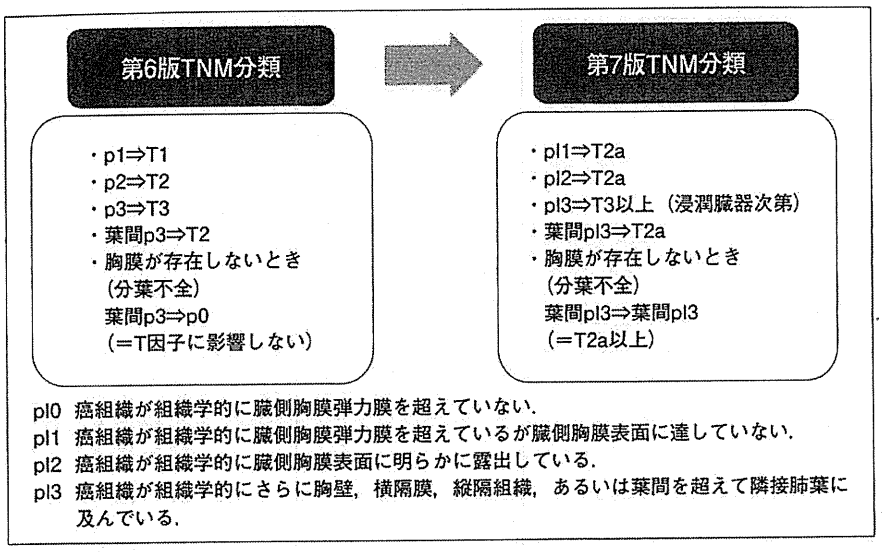

図1
胸膜浸潤に関する変更点のまとめ

III N因子

N因子に関しての定義の変更はなかったが，リンパ節マップが改訂となり，解剖学的な境界線も変更がなされた．今回のリンパ節マップ改訂は予後解析を基にしたものではなく，解剖学的に境界を明瞭化し，各国で使用されていたそれぞれ異なるリンパ節マップの統一を図ったものといえる．リンパ節部位の規定を表2[1]に示す．

1 鎖骨上窩リンパ節（#1R，#1L）

これまでリンパ節マップに示されていなかった鎖骨上窩領域が#1（上縁は気管輪状軟骨下縁，下縁は左右鎖骨および正中では胸骨柄上縁）と規定された．これは後述する上縦隔リンパ節が4領域から3領域に整理されたことで，すべての領域の番号が1つ繰り上がるという混乱を防ぐ意味合いが強かったと思われる．

2 上縦隔リンパ節（#2R，#2L，#3a，#3p，#4R，#4L）

重要な変更点は従来の「#2，#4」との境界が不明瞭であった「#3」が除かれて（#3aと#3pは残存）今回#2，#4に2分されたことである．また#2，#4の左右の境界は正中線ではなく気管左縁に設定された（図2）．これはリンパ節郭清を行う際の外科的解剖学が大きく反映されたものと思われる．つまり，#2Rと#4Rの郭清は右胸腔からアプローチを行うが，単に気管正中線が判断しにくいという理由だけではなく，気管正中を越え気管左縁までの郭清は容易な半面，左胸腔からのアプローチでは大動脈が障害となり気管前面に到達できないという解剖学的な理由である．

3 気管分岐下リンパ節

従来のNaruke mapで#7と#10に分けられていた気管分岐下リンパ節は，今回の改訂ですべて#7に変更された．これに伴い従来の#10リンパ節のうち，主気管支の縦隔側で#10とされていた部位に転移があった場合はN1からN2に変更となり，対側#10リンパ節はN3からN2へ変更される．過去の手術例で郭清した#10リンパ節の詳細な部位を記録していない症例などは病期の再分類に苦慮する可能性が考えられる（図2）．

IV M因子

1 悪性胸水・心嚢水

今回の改訂で悪性胸水・心嚢水はT4からM1aに変更された．以前より癌性胸膜炎・心嚢炎のあるstageⅢB症例（wet ⅢB）は，ほかのⅢB症例と異なり放射線治療の対象外とされ，予後もstageⅣに近いと考えられていた．IASLCのデータベース検討でT4M0を胸膜播種とそれ以外の症例に分けた結

表2　リンパ節の部位の規定

大分類	略語	小分類 (リンパ節部位の命名)	リンパ節の部位の規定（以下の領域に存在するリンパ節を指す）
鎖骨上窩リンパ節	#1R, #1L	鎖骨上窩リンパ節	上縁：気管輪状軟骨下縁 下縁：左右鎖骨および正中では胸骨柄上縁 右側：1R，左側：1L（左右は正中線で分ける）
上部縦隔リンパ節	#2R	右上部気管傍リンパ節	上縁：右肺尖，胸膜頂および正中では胸骨柄上縁 下縁：気管と左腕頭静脈尾側縁の交点 左側縁：気管左側縁
	#2L	左上部気管傍リンパ節	上縁：左肺尖，胸膜頂および正中では胸骨柄上縁 下縁：大動脈弓上縁 右側縁：気管左側縁
	#3a	血管前リンパ節	右側：上縁：胸膜頂，下縁：気管分岐部，前縁：胸骨後面，後縁：上大静脈前縁 左側：上縁：胸膜頂，下縁：気管分岐部，前縁：胸骨後面，後縁：左総頸動脈
	#3p	気管後リンパ節	上縁：胸膜頂，下縁：気管分岐部までの気管後壁より後ろに位置するリンパ節
	#4R	右下部気管傍リンパ節	右側気管傍および気管前に存在するリンパ節 上縁：気管と左腕頭静脈尾側縁の交点 下縁：奇静脈弓尾側縁 左側縁：気管左側縁
	#4L	左下部気管傍リンパ節	気管左側縁と動脈管索の間に存在するリンパ節 上縁：大動脈弓上縁 下縁：左主肺動脈の上内側周囲縁 右側縁：気管左側縁 左側縁：動脈管索
大動脈リンパ節	#5	大動脈下リンパ節	動脈管索の外側に存在するリンパ節 上縁：大動脈弓下縁 下縁：左主肺動脈の上外側周囲縁
	#6	大動脈傍リンパ節	上行大動脈，大動脈弓の前方および外側に存在するリンパ節 上縁：大動脈弓上縁の接線レベル 下縁：大動脈弓下縁
下部縦隔リンパ節	#7	気管分岐下リンパ節	気管分岐部と左右気管支に囲まれた領域のリンパ節 上縁：気管分岐部 下縁：左側では左下葉気管支の上縁，右側は中間気管支幹下縁
	#8	食道傍リンパ節	食道に接して存在するリンパ節（気管分岐部リンパ節を除く） 上縁：左側では左下葉気管支の上縁，右側は中間気管支幹下縁 下縁：横隔膜
	#9	肺靱帯リンパ節	肺靱帯内にあるリンパ節 上縁：下肺静脈 下縁：横隔膜
肺門リンパ節	#10	主気管支周囲リンパ節	主気管支の周囲および主肺動脈，肺静脈中枢側周囲に存在するリンパ節 上縁：右側では奇静脈下縁，左側では左主肺動脈上側周囲縁 下縁：左右葉間
	#11	葉気管支間リンパ節	葉気管支間に存在するリンパ節 上中葉間リンパ節（#11s）：右上葉気管支と中間気管支幹との間のリンパ節 中下葉間リンパ節（#11i）：中下葉支との間のリンパ節
肺内リンパ節	#12	葉気管支周囲リンパ節	葉気管支周囲に存在するリンパ節
	#13	区域気管支周囲リンパ節	区域気管支周囲に存在するリンパ節
	#14	亜区域気管支周囲リンパ節	亜区域気管支周囲またはさらに末梢の気管支周囲に存在するリンパ節

〔参考文献1）より引用改変〕

果，それぞれ生存期間中央値（median survival time：MST）が10カ月と13カ月，1年生存率は45%と53%，5年生存率は6%と16%といずれも胸膜播種症例が有意に予後不良（$p>0.0001$）であることから[7]，今回の変更は妥当と考えられる．肺癌患者で胸水や心嚢水を認めた場合，通常は腫瘍によるものと考えるが，血性や滲出性でなく細胞診が複数回陰性である場合は非腫瘍性のものと判断されM0とすることが記載されている．

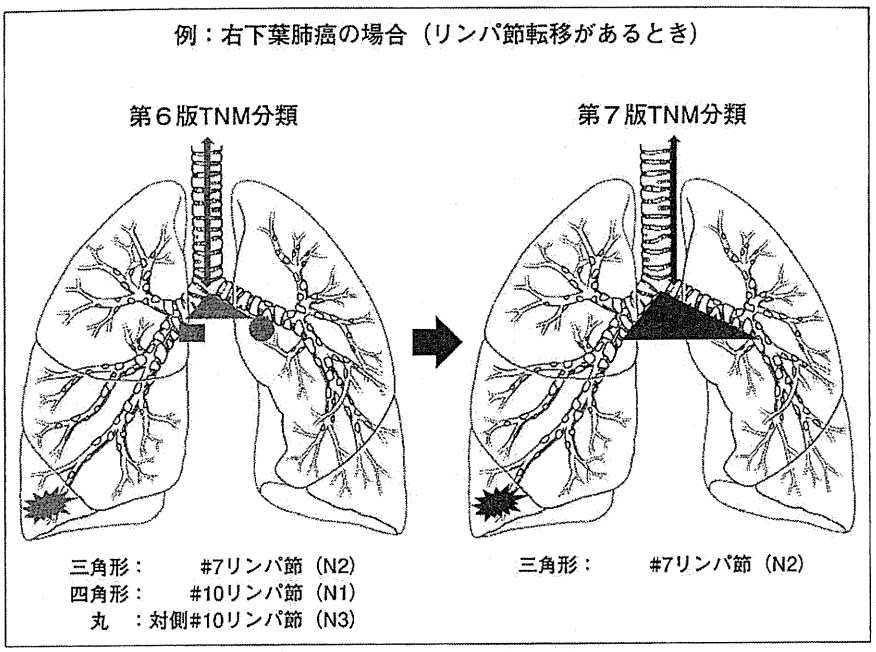

図2
外科解剖学に基づくリンパ節マップの主な変更点
矢印：上縦隔リンパ節の左右の境界線

2　遠隔転移

遠隔転移はM1bとなり，対側肺転移，悪性胸水・心嚢水のM1aとは別に定義された．いずれもstage Ⅳという点では変わりはないが，遠隔転移症例は対側肺転移や悪性胸水・心嚢水貯留症例と比較して予後不良という報告[7]を受けての分類と考えられる．

Ⅴ　おわりに

今回の第7版TNM分類は，以前のものと比べてとても煩雑化しており覚えにくいという問題点が指摘されているが，10万例超という多数の症例の解析を基に細分化が行われ，より予後を反映したものとなっている．また第6版TNM分類があまり用いられなかった小細胞癌でも，第7版TNM分類では1万例以上の小細胞癌のデータが集積されており，積極的にTNM分類を行うことで治療法の検討や予後の予測などに有用であると考えられる．今回の改訂は，あくまで解剖学的な因子に基づいて病期分類が作成されたが，今後は脈管浸潤の有無や組織の特徴，分子マーカーなどの病理学的な予後因子が導入されるかが検討事項となるであろう．

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Klotho is a novel biomarker for good survival in resected large cell neuroendocrine carcinoma of the lung

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ABSTRACT

Background: In terms of prognosis, large cell neuroendocrine carcinoma (LCNEC) differs distinctively from other non-small cell lung cancers, with the prognosis of LCNEC being poor, even for early-stage disease. Improvements in survival require a biomarker capable of defining a subset of patients destined to do poorly so that these patients can be targeted for additional therapies, including chemotherapy. 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 immunohistochemical expression of Klotho can predict survival patients with resected LCNEC.
Methods: The histological characteristics of patients receiving an initial diagnosis of LCNEC ($n=30$) at Tokyo Medical University Hospital were retrospectively reviewed, and multiple variables including stage, lymphangioinvasion, lymph node status and the expression of Klotho as identified using an immunohistochemical analysis, were assessed.
Results: Immunostaining for Klotho was mostly cytoplasmic, and Klotho expression was seen in 10 patients (33.3%) but not in 20 patients (66.7%). The expression of Klotho was significantly associated with a good outcome of resected patients with LCNEC and Klotho(−) was associated with increased LCNEC risk by multivariate analysis (hazard ratio 4.92, 95% confidence interval 1.04–23.24, $p=0.044$). Neither lymph node status nor lymphangioinvasion were significantly associated with a poor survival. However, among patients without lymph node metastasis or angioinvasion, the survival benefit of Klotho expression in the primary tumor was significantly higher, compared with that of patients without Klotho expression.
Conclusion: Klotho staining provides a new biomarker for a good outcome in patients with LCNEC, especially among patients without lymph node metastasis or lymphangioinvasion.

1. Introduction

In the World Health Organization (WHO) classification, large cell neuroendocrine carcinoma (LCNEC) is categorized as a variant of large cell carcinoma [1,2]. When LCNEC is combined with adenocarcinoma, squamous cell carcinoma, or giant cell carcinoma, it is categorized as combined LCNEC. LCNEC reportedly represents about 2–3% of all lung cancers, and the prognosis of LCNEC is poor, even for early-stage disease; the prognosis of stage I LCNEC is, in fact, poorer than that of the same stage of other non-small cell lung cancers [3–6]. Asamura et al. reported a study with a large sample size that was conducted in a retrospective, multi-institutional setting and included a critical review of the histological findings; the authors concluded that no prognostic difference was noted between LCNEC and small cell lung cancer (SCLC) [7].

Recently, we examined the clinical response of LCNEC to perioperative adjuvant chemotherapy and reported that perioperative chemotherapy may improve survival in patients with resected LCNEC [8]. Therefore, a biomarker capable of predicting either a good or poor outcome among patients with resected LCNEC is needed [9,10]. New molecular biomarkers, such as the excision repair cross-complementation group 1 protein, have attracted considerable interest with regard to the prediction of outcome among early-stage patients who have undergone resection [11].

The insulin-like growth factor (IGF) pathway is involved in the normal control of fetal development, tissue growth, and metabolism [12–14]. The IGF pathway has been implicated in the induction and maintenance of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [15]. Dziadziuszko et al. reported that elevated plasma levels of IGF-1 have been associated with

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an increased risk of lung cancer [16]. An immunohistochemical analysis demonstrated that intracellular IGF binding protein was highly expressed in lung adenocarcinomas, and the expression of IGF-messenger RNA-binding protein was higher in LCNECs than in SCLCs [17,18]. A number of IGF receptor inhibitors, including monoclonal antibodies and small molecule inhibitors, are currently undergoing testing in clinical trials [19,20].

Recently, the Klotho gene, which is a 1014-amino acid single-pass transmembrane protein first identified as an anti-aging gene, has been shown to function as an inhibitor of IGF pathways, and is a potential tumor suppressor in breast cancer [21–25]. Therefore, we hypothesized that the *Klotho* gene may play an important role in regulating cell growth and LCNEC proliferation and may predict the survival of patients with LCNEC. In this study, we investigated whether the immunohistochemical expression of Klotho can predict survival in resected LCNEC.

2. Materials and methods

2.1. Patient selection

Of 1400 patients who underwent surgical resection for primary lung cancer between January 1999 and December 2004 at our institution, 30 patients with LCNEC were enrolled in this study. According to the histological typing of lung and pleural tumors in the WHO International Histological Classification of Tumors, 3rd edition, LCNEC is classified as a variant of large cell carcinoma (LCC) [1–4]. Because previous studies reported a similar prognosis for pure and combined LCNEC, both forms are included in the present study [5–7]. An immunohistochemical analysis was performed to confirm the neuroendocrine differentiation of the tumors. For a definitive diagnosis of LCNEC, formalin-fixed paraffin sections were stained using a panel of neuroendocrine markers that included chromogranin A (CGA) (1:1500, Dako), synaptophysin (SYN) (1:100, Dako), and neural cell adhesion molecule (NCAM) (1:50, Novacastra), using standard methods [5–8]. All the histological specimens were diagnosed by experienced pathologists in the Department of Pathology, Tokyo Medical University Hospital. This study was conducted with the approval of the Ethical Committee of Tokyo Medical University.

2.2. Immunostaining for Klotho

Immunohistochemical staining was performed on 4-μM formalin-fixed, paraffin-embedded tissue sections [6,7,9]. 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-Klotho antibody (kindly provided by Prof. Nabeshima, Kyoto Univ.). Antibody staining was considered positive if >10% of the tumor cells were stained, based on the use of a 10% cutoff level in several previous studies [26]. All the slides were examined by two observers without knowledge of the patients' clinical data [24–26].

2.3. Statistics

Clinical information was extracted from the medical records. The disease stage was based on the TNM classification using the International Union Against Cancer (UICC) staging system. Statistical analyses were performed using SPSS for Windows. The Kaplan–Meier method was used to determine survival [26–29].

Table 1
Clinicopathological characteristics of 30 patients with LCNEC.

Patient characteristics	No. of patients
No. of cases (%)	30
Age (mean)	40–82 (69.8)
Gender	
Male	27
Female	3
Surgical procedure	
Lobectomy	29
Pneumonectomy	1
p-Stage	
IA	2
IB	12
IIA	3
IIB	3
IIIA	4
IIIB	5
IV	1
Perioperative chemotherapy	
Yes	18
No	12
Alive	
Yes	12
No	18

Overall survival was defined as the time (in months) from the date of surgery until the time of death. We assessed the univariate effect of each variable on survival using the log-rank test for categorical predictors or the univariate Cox model for continuous predictors [26–29]. Variables were considered significant in the univariate analyses. Lymph node invasion, Klotho expression, and lymphangio invasion were candidates in this final model of 0.05 for entry into the model.

3. Results

The clinicopathologic characteristics of the patients are listed in Table 1. Their mean age at the time of surgery was 69.8 years (range, 40–82 years). Twenty-seven patients were men, and 3 patients were women. The surgical procedures that were performed included 29 lobectomies and 1 pneumonectomy. The distribution of pathological staging was 2 (6.7%) patients with stage IA, 12 (40%) with stage IB, 3 (10%) with stage IIA, 3 (10%) with stage IIB, 4 (13.3%) with stage IIIA, 5 (16.6%) with stage IIIB and 1 (3.3%) with stage IV. The median follow-up period was 45 months (range, 12–96 months); during the study period, 18 patients (60%) died and 12 patients survived for more than 60 months. We examined Klotho expression using an immunohistochemical analysis. As Fig. 1 shows, immunostaining for Klotho was mainly observed in the cytoplasm, and Klotho expression was seen in 10 patients (33.3%) but not in 20 patients (66.7%). We investigated whether Klotho staining might predict a good outcome. Fig. 2 shows the Kaplan–Meier survival curve for Klotho-positive patients (10 patients) and for Klotho-negative patients (20 patients). The expression of Klotho was significantly associated with survival in patients with resected LCNEC ($p = 0.013$). Klotho-negative patients with LCNEC had a worst overall survival and Klotho expression was an independent prognostic factor by multivariate analysis (hazard ratio = 4.92; 95% confidence interval: 1.04–23.24; $p = 0.044$) (Table 2). Neither lymph node status nor lymphangioinvasion were significantly associated with poor survival, as shown in Figs. 3 and 4. In this study, lymph node status was not a prognostic factor by multivariate analysis (hazard ratio = 1.99; 95% confidence interval: 0.72–5.47; $p = 0.185$) (Table 2). Among the nine patients with pathological stage IB, six patients without Klotho expression died and three patients with Klotho expression were still alive. However, among the 14 patients with a negative lymph node status, including

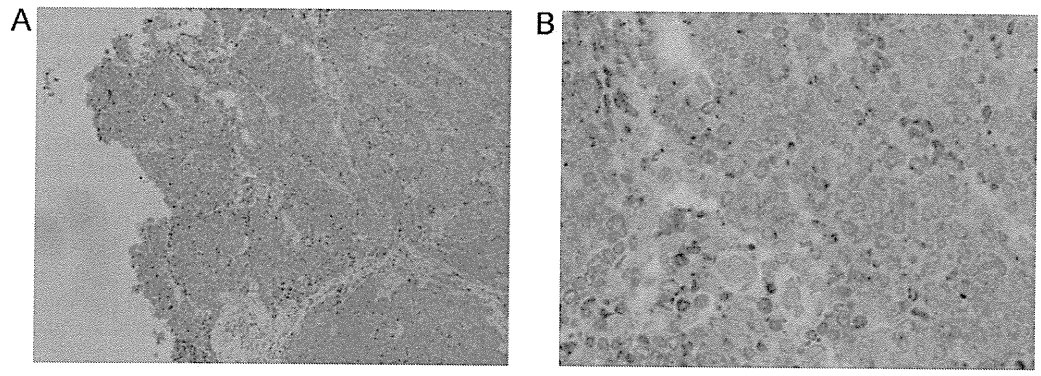

Fig. 1. Immunohistochemical staining with anti-Klotho antibody (KM2906) in resected LCNEC (A, 40×; B, 400×).

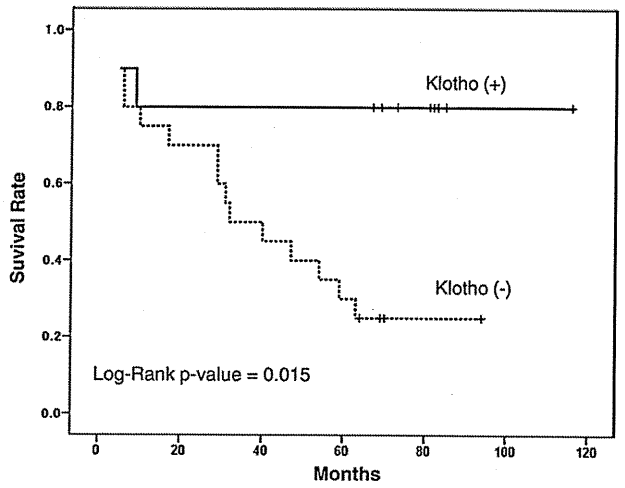

Fig. 2. Kaplan–Meier survival plot showing the survival of 30 patients with LCNEC who were positive ($n=10$) or negative ($n=20$) for Klotho expression. Patients with LCNEC and Klotho (+) expression have a significantly better prognosis than those with Klotho (−) expression ($P=0.015$).

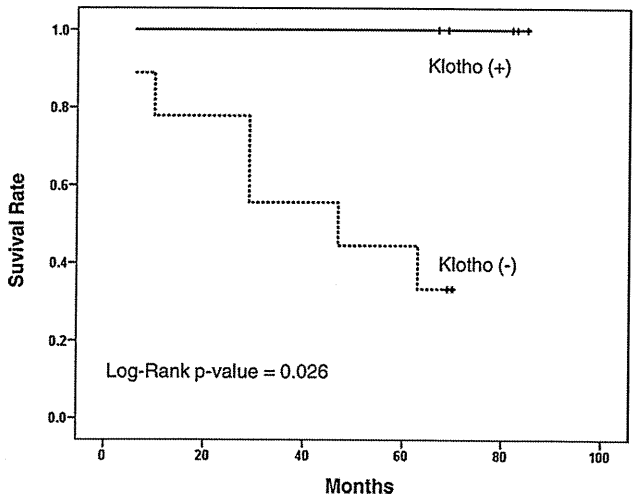

Fig. 3. Kaplan–Meier survival plot showing the survival of 14 patients with LCNEC but without lymph node metastasis. A significant difference in survival was observed between patients with Klotho (+) expression ($n=5$) and those with Klotho (−) expression ($n=9$). The prognosis of the patients with Klotho (+) expression was better than that of the patients with Klotho (−) expression ($P=0.026$).

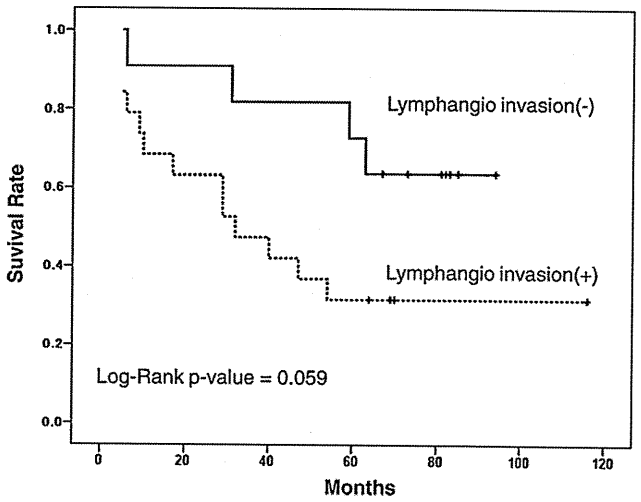

Fig. 4. Kaplan–Meier survival plot showing the survival of 30 patients with LCNEC and lymphangioinvasion (+) ($n=19$) or lymphangioinvasion (−) ($N=11$). Lymphangioinvasion did not significantly affect survival ($P=0.059$).

those with p-stage IA, IB, and IIA, 6 patients died and 8 patients were still alive. Of these 14 patients, 5 patients had Klotho expression and were alive and 9 patients did not have Klotho expression. Fig. 3 shows that among the 14 patients without lymph node metastasis, the survival curve of patients with Klotho expression in their primary tumors was significantly higher than that of patients without Klotho expression.

Vascular or lymphatic vessel invasion have both been suggested as potential markers of a poor outcome among stage IA

Table 2
Parameters related to overall survival (multivariate analysis).

	Cases	HR (95% CI)	P^a value
Klotho expression			
Positive	10	1	
Negative	20	4.92 (1.04–23.24)	0.044
Lymph node			
Meta (−)	14	1	
Meta (+)	16	1.99 (0.72–5.47)	0.185
Lymphangioinvasion			
Absent	11	1	
Present	19	1.93 (0.61–6.13)	0.264
Perioperative chemotherapy			
Yes	20	1	
No	10	1.98 (0.73–5.36)	0.181

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

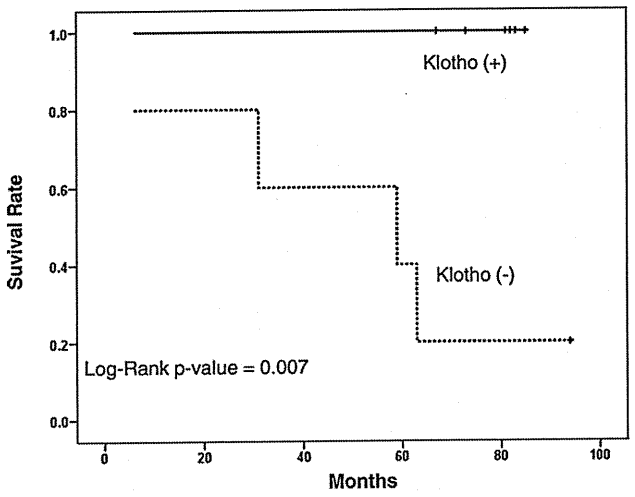

Fig. 5. Kaplan–Meier survival plot showing the survival of 11 patients with LCNEC but without lymphangioinvasion. A significant difference in survival was observed between the patients with Klotho (+) expression ($N=6$) and those with Klotho (−) expression ($N=5$). The prognosis of the patients with Klotho (+) expression was better than that of those with Klotho (−) expression ($P=0.007$).

patients, but the results have been controversial and inconsistent [7–10]. In this study, no significant difference in outcome was observed between patients with lymphangioinvasion and those without lymphangioinvasion, as shown in Fig. 4. Therefore, we focused on patients without lymphangioinvasion. As shown in Fig. 5, among the lymphangioinvasion-negative patients, the survival rate was significantly higher in those with Klotho expression in their primary tumors than in those without Klotho expression. These results suggest that the survival of patients with LCNEC who are lymph node negative or lymphangioinvasion negative might be strongly influenced by the presence of Klotho expression, and Klotho immunostaining was a highly significant prognostic marker for patients with resected LCNEC.

4. Discussion

Patients with LCNEC have a very poor prognosis, and Asamura et al. reported that the 5-year survival rates of patients with all disease stages were 40.3% for LCNEC and 35.7% for SCLC, with no significant difference noted between LCNEC and SCLC [7]. Considering the 5-year survival rate of stage I non-small cell lung cancer, the LCNEC histology is associated with a dismal prognosis, and LCNEC has almost the same prognosis as SCLC [7]. These two histologies also share similar clinicopathological features, such as a smoking history and male prevalence, and incomplete resection, and nodal involvement are significant prognostic factors for both LCNEC and SCLC.

Recently, the potential uses of neuronatin (NNAT), cytokeratin 7 (CK7), CK18, E-cadherin and β-catenin as differential markers for LCNEC and SCLC have been reported [7–11]. The existence of prognostic factors capable of predicting the survival rate of patients with LCNEC after curative resection remains uncertain. In the present study, although the number of resected LCNEC was relatively small, our data indicate that Klotho expression may predict the good outcome of patients with LCNEC and that Klotho expression is a prognostic factor after curative resection, and our data need to be verified in more larger cases.

Wolf et al. identified Klotho as a potent tumor suppressor gene in breast cancer, and Klotho expression may serve as a predictor of breast cancer risk among *BRCA 1* mutation carriers [24]. Moreover, Wolf et al. reported that Klotho overexpression specifically reduces the colony formation of breast cancer cell, and most of Klotho's growth inhibitory activities in the breast are mediated by the secreted protein [24,25]. Klotho expression may play an important role in improving the treatment outcome of patients with LCNEC.

We retrospectively analyzed the association between perioperative chemotherapy and the survival benefit of LCNEC and reported that perioperative chemotherapy is needed to improve the survival of patients with resected LCNEC [8]. Surgical resection alone did not appear to improve the prognosis of patients with LCNEC as previous reports [30,31]. In the present study, no significant difference between Klotho expression and the survival benefit of perioperative chemotherapy was noted for patients with LCNEC. Although the number of patients with LCNEC is relatively small and randomized controlled trials demonstrating a survival benefit of adjuvant chemotherapy are difficult to conduct, the association between Klotho expression and the survival benefit of perioperative chemotherapy for LCNEC should be further evaluated in larger multi-institutional trials.

In summary, we demonstrated that Klotho expression has a significant prognostic value in resected LCNEC of the lung. In the future, the evaluation of this marker may improve the personalization of the treatment in this aggressive histotype of lung cancer.

Conflict of interest statement

There was no financial support for the authors nor does any author have a financial relationship with a commercial entity that has an interest in this manuscript.

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Randomized Phase II Study of Two Schedules of Carboplatin and Gemcitabine for Stage IIIB and IV Advanced Non-Small Cell Lung Cancer (JACCRO LC-01 Study)

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Key Words
Lung cancer · Randomized phase II study · Gemcitabine · Carboplatin · Thrombocytopenia

Abstract
Background: Gemcitabine combined with carboplatin (CG) is one of the regimens used widely for advanced non-small cell lung cancer. Improvement in its toxicity may result in good clinical outcomes. ***Methods:*** A new schedule of gemcitabine and carboplatin (CG8) was compared with the standard one (CG1). Both are 3-weekly regimens, but carboplatin is administered on day 1 in CG1 and on day 8 in CG8. ***Results:*** The response rate of CG1 was 29.2%, which was higher than that of CG8 (22.2%). Median survival times in CG1 and CG8 were 348 and 455.5 days, respectively. Grade ≥3 leukopenia, thrombocytopenia and anemia were observed in 56.0, 72.0 and 36.0% of patients with CG1 and in 33.3, 25.9 and 14.8% of patients with CG8, respectively. Whereas grade ≥3 elevation of asparatate aminotransferase, alanine aminotransferase and alkaline phosphatase was seen mainly in CG8, grade ≥3 non-hematologic toxicities such as febrile neutropenia, infection, appetite loss, diarrhea and eruption were observed only in CG1. ***Conclusion:*** CG1 is superior in response rate, but CG8 shows improved toxicities and a tendency of prolonged survival.

Introduction

Cytotoxic chemotherapy still plays a pivotal role in the management of advanced non-small cell lung cancer (NSCLC), whereas recently, the combination of platinum doublets and molecular targeted drugs has been extensively studied. Among several platinum doublets, the combination of carboplatin and gemcitabine (CG) is a popular regimen. Carboplatin is commonly used in a practical setting compared with cisplatin, although the antitumor effect of carboplatin is suggested to be somewhat inferior to cisplatin [1–3]. Several studies have shown that platinum doublets with 3rd-generation cytotoxic drugs were similar in antitumor activities but different in toxicities [4]. A recent trial showed that pemetrexed/cis-

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platin produced better survival compared with gemcitabine/cisplatin in NSCLC with non-squamous (Non-Sq) histology [5]. In contrast, Grønberg et al. [6] showed that the superiority in survival of pemetrexed over gemcitabine is not clear when carboplatin was combined with these drugs. Hematologic toxicities of CG were more severe than those of pemetrexed/carboplatin: grade ≥3 leukopenia 46 and 23% (p = 0.001), neutropenia 51 and 40% (p = 0.024) and thrombocytopenia 56 and 24% (p = 0.001) in CG and pemetrexed/carboplatin, respectively. More patients in the CG arm received transfusions of red blood cells and platelets. If hematologic toxicities of CG regimens are improved, its application will be extended in the 1st-line treatment for Sq NSCLC or as an alternative of pemetrexed/carboplatin for Non-Sq NSCLC.

Clinical trials including CG regimens initially adopted a 4-week regimen. Subsequently, 3-week schedules in which gemcitabine was administered on days 1 and 8 were evaluated. In phase II studies, 3-week regimens showed comparable efficacy with the 4-week schedules [7]. In the 4-week arm, carboplatin at an area under the curve (AUC) of 5 mg/ml × min was administered on day 8 and gemcitabine 1,000 mg/m^2 was given on days 1 and 8. In the 3-week arm, carboplatin at AUC 5 was given on day 1, and gemcitabine at 1,000 mg/m^2 was administered on days 1 and 8. Obasaju et al. [8] compared 3- and 4-week schedules of CG in 472 advanced NSCLC patients. Although statistically not significant, 2% complete responses and 38% partial responses produced by the 3-week schedule are better than no complete response and 23% partial responses by the 4-week schedule. In contrast, the frequency of grade 3 or 4 thrombocytopenia, which is particularly problematic in CG, was 14% in the 3-week schedule and 8% in the 4-week schedule. A Japanese comparative phase II study, where 3-week schedule CG was compared with the combination of gemcitabine and vinorelbine, also showed a high incidence of dose reduction and early withdrawal due to myelosuppression, mainly thrombocytopenia, in CG [9].

These results indicate that hematologic toxicities of the 3-week CG regimen should be improved. We already reported a new regimen, in which gemcitabine of 1,000 mg/m^2 was administered on days 1 and 8 and carboplatin of AUC 5 on day 8 every 21 days. A schedule-dependent synergistic effect of gemcitabine combined with pemetrexed was reported in the treatment of NSCLC cell lines [10]. A similar schedule-dependent synergy may be observed in the CG combination. In a preceding phase II trial with 31 patients with stage IIIB or IV NSCLC, the response rate was 22.6%, including one complete response, and median time to progression and median survival time were encouraging, i.e. 161 and 454 days, respectively [11]. In this regimen, the criteria to start new cycles and those to perform day 8 infusion were specially defined in order to improve myelosuppression and to maintain dose intensity: white blood cell count (WBC) ≥2,500/mm^3 and platelet count (Plt) ≥750,000/mm^3 to start new cycles and WBC ≥3,000/mm^3 and Plt ≥100,000/mm^3 to perform day 8 infusion. As reported previously, these criteria were proved to work well, resulting in grade 3/4 thrombocytopenia only in 2 patients (6.5%; one grade 3 and one grade 4). Since this phase II study was performed in a single institution, we have compared the 3-week CG regimen that we developed with the standard 3-week CG regimen in a multi-institutional randomized phase II study.

Patients and Methods

Eligibility

Patients were eligible for study participation when they met the following criteria: age <75 years; histologic or cytologic diagnosis of NSCLC; clinical stage IIIB not amenable to curative treatment or stage IV by the Union for International Cancer Control TNM classification version 6; first-line treatment; Eastern Cooperative Oncology Group performance status 0–1; measurable disease in Response Evaluation Criteria in Solid Tumors (RECIST); adequate bone marrow reserve (neutrophil count ≥1,500/mm^3, Plt ≥10 × 10^4/mm^3, hemoglobin ≥9.0 g/dl); acceptable hepatic (serum bilirubin <1.5 mg/dl, transaminases less than twice the upper limit of normal) and renal function (normal serum creatinine and creatinine clearance determined by Cockcroft equation ≥50 ml/min), and a life expectancy of at least 3 months. Patients were excluded from the study when they met one of the following conditions: active uncontrolled infection; unstable concomitant disease (ischemic heart disease, hypertension, arrhythmia, cirrhosis and diabetes mellitus); active concomitant malignant disease; massive effusion; concomitant interstitial lung disease; superior vena cava syndrome; brain metastasis, and pregnancy or breastfeeding. Written informed consent was obtained from all patients.

Study Design

The eligible patients were randomized to the CG1 or the CG8 arm. In the CG1 arm, carboplatin of AUC 5 calculated using the Calvert formula with creatinine clearance evaluation by the Cockcroft equation and gemcitabine of 1,000 mg/m^2 were administered as an intravenous injection on day 1 and on days 1 and 8, respectively (CG1). In the CG8 arm, the same doses of CG were administered as an intravenous injection on day 8 and on days 1 and 8, respectively (CG8). Treatment was repeated every 3 weeks. The two arms are similar, but developed independently. Therefore, we adopted independent hematologic criteria suitable for each arm to start new cycles and to perform day 8 infusion: in the CG1 arm, WBC ≥3,000/mm^3 and Plt ≥10 × 10^4/mm^3 were required to start new cycles, and WBC ≥2,000/mm^3 and Plt ≥100,000/mm^3 were necessary to perform day 8 infusion. In the CG8 arm, corresponding hematologic criteria were the same as

those in our preceding phase II study described in the Introduction section: WBC ≥2,500/mm^3 and Plt ≥750,000/mm^3 to start new cycles, and WBC ≥3,000/mm^3 and Plt ≥100,000/mm^3 to perform day 8 infusion. When one of these criteria was not met, treatment was skipped. A dose reduction of up to two times was permitted in the case of a leukocyte count <1,000/mm^3, Plt <25,000/mm^3, febrile neutropenia, grade >2 non-hematologic toxicity, or skip of day 8 administration in the preceding cycle. The dose of gemcitabine was reduced to 800 mg/m^2 in the first dose reduction and that of carboplatin to AUC 4 in the second one. After withdrawal from the study, subsequent treatment was decided by the investigator.

This study was performed by JACCRO (Japanese Cancer Clinical Research Organization) as an LC-01 study.

Evaluation of Toxicity and Response

Toxicity was scored every 3 weeks during treatment and every month thereafter according to the National Cancer Institute Common Toxicity Criteria version 2.0. Response was evaluated every 4 weeks during treatment and every 6 months until disease progression thereafter, according to RECIST criteria [12]. Brain MRI, chest CT scan and abdominal CT scan were performed at any time if assessment for disease progression was necessary. Objective responses were required to be confirmed after at least 4 weeks.

Endpoint and Statistical Analysis

The primary endpoint of this study was the response rate. Secondary endpoints included overall survival, toxicities, completion rate of 1–3 cycles, and dose intensity during 1–3 cycles. If the threshold response rate and the expected response rate were to be 20 and 35%, respectively, the study has 90% power to detect the better arm with 90% confidence using Simon's selection design when 29 patients were included in each arm. The Kaplan-Meier method was used to plot overall survival.

Results

Patient Characteristics

From February 2005 to April 2007, 55 patients were enrolled in the study. Protocol amendment was done to prolong accrual time because of slow accrual, but the entry of the patients was finally stopped before completing the planned accrual of 60 patients. One patient was ineligible because of preceding chemotherapy, and 2 patients who had been allocated to the CG1 arm received CG8. Excluding these 3 patients, 25 patients in the CG1 arm and 27 patients in the CG8 arm were analyzed. Patient backgrounds are shown in table 1. Although ages, stages and histology are well balanced between the two arms, the ratios of men and the patients with a performance status of 1 seem to be slightly higher in the CG1 arm.

Treatment Delivery

The median number of the cycles administered was 3 in both arms. Whereas more patients underwent the 2nd to 4th cycle in the CG8 arm compared with the CG1 arm, the percentage of the patients who received the 5th and 6th cycle was higher in the CG1 arm. As shown in table 2, 52.0% of the patients in the CG1 arm and 66.7% in the CG8 arm received at least 3 cycles of chemotherapy, and 40.0% of the patients in the CG1 arm and 33.3% in the CG8 arm did without skips of administration schedule. Skipping administration on day 8 occurred in 36 and 48.1% in CG1 and CG8, and dose reduction was necessary in 56.0 and 40.7% in CG1 and CG8, respectively. Reflecting these modifications, the relative dose intensity of gemcitabine was 67.2% of the planned dose in CG1 and 74.4% in CG8, and that of carboplatin was 76.3% in CG1 and 68.1% in CG8 in the first 3 cycles (table 2).

Table 1. Patient characteristics

	Arm A	Arm B
Patients	25	27
Age, years		
Mean	59.2	61.4
Range	40–74	40–73
Men/women	19/6	15/12
Performance status 0/1	10/15	14/13
Stage (IIIb/IV/after operation)	5/18/2	4/21/2
Histology (Ad/Sq/La)	22/2/1	21/4/2

Ad = Adenocarcinoma; Sq = squamous cell carcinoma; La = large cell carcinoma.

Table 2. Treatment delivery and dose intensity

	Arm A	Arm B
Completion of 3 cycles, %		
Overall	52.0	66.7
Without skips	40.0	33.3
With skips	12.0	33.3
Dose intensity, % of planned dose		
Carboplatin	76.3	68.1
Gemcitabine	67.2	74.4

Efficacy Results

The response rate in the CG1 arm was 29.2% and is higher than that in the CG8 arm (22.2%; table 3). The response rates of both arms exceeded the threshold of 20%, but that of the better one did not reach the expected value of 35%. The rate of progression was higher in CG1 than

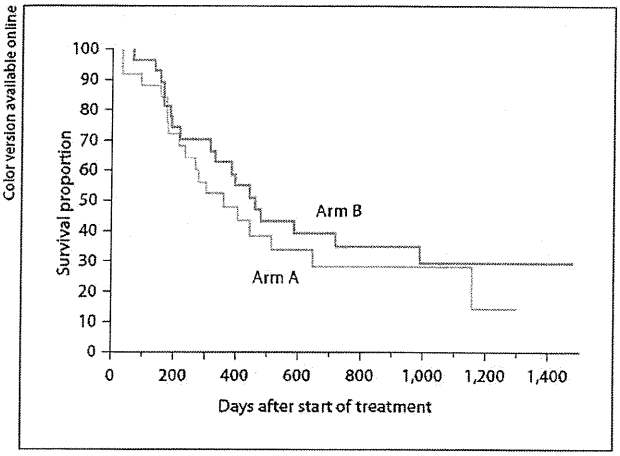

Fig. 1. Survival curves. Survival curves of arms A and B were drawn by the Kaplan-Meier method. The difference is not statistically significant (p values are 0.40 and 0.41 by the log-rank test and Wilcoxon test, respectively).

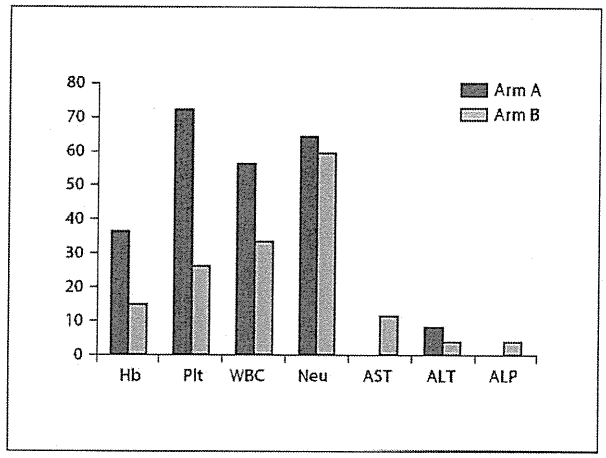

Fig. 2. Hematologic toxicity and abnormal blood chemistry of grade 3 and 4. Toxicities in blood hemoglobin value (Hb), WBC and neutrophil counts (Neu) are shown. AST = Asparatate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase.

Table 3. Response and survival

	Arm A	Arm B
Response rate, %	29.2	22.2
CR	4.0	0
PR	24.0	22.2
SD	40.0	51.8[1]
PD	28.0	25.9
NE	4.0	0
Median survival time[2], days	348	455.5

CR = Complete response; PR = partial response; SD = stable disease; PD = progressive disease; NE = not evaluable.
[1] Including one unconfirmed partial response.
[2] p = 0.40 by the log-rank test; p = 0.41 by the Wilcoxon test.

that in CG8. Overall survival curves were shown in figure 1. Median overall survival time was 348 and 455.5 days in the CG1 and CG8 arm, respectively.

Toxicity

Toxicity profiles are summarized in figure 2 and table 4. Hematologic toxicities were generally milder in CG8 than in CG1: grade 3/4 leukopenia was observed in 56.0 and 33.3% of the patients, grade 3/4 thrombocytopenia in 72.0 and 25.9%, and grade 3/4 anemia (hemoglobin) in 36.0 and 14.8% in the CG1 and CG8 arm, respectively, whereas the frequency of grade 3/4 neutropenia was comparable between the two arms (64.0 and 59.2%, respectively). Grade 4 thrombocytopenia occurred in 4.0% in CG1, whereas no grade 4 thrombocytopenia was observed in CG8. Abnormal blood chemistry was sporadically observed but not severe in both arms. Table 4 summarizes non-hematologic toxicities of grade ≥3 except those in blood chemistry. Whereas the grade ≥3 elevation of asparatate aminotransferase, alanine aminotransferase and alkaline phosphatase was seen mainly in CG8, grade ≥3 non-hematologic toxicities such as febrile neutropenia, infection, appetite loss, diarrhea and eruption were observed only in CG1.

Discussion

Recently, there has been progress in medical treatment for advanced NSCLC, incorporating molecular targeted drugs such as epidermal growth factor receptor tyrosine kinase inhibitors and bevacizumab, an angiogenesis inhibitor. However, cytotoxic drugs are still playing a pivotal role acting as themselves or as platforms with which molecular targeted drugs are combined. Less toxic chemotherapy regimens will have the advantages to maintain the quality of life of the patients

Table 4. Non-hematologic toxicities

	Arm A, %		Arm B, %	
	G2	G3	G2	G3
Febrile neutropenia	–	4.0	–	0
Infection	0	8.0	0	0
Body weight loss	0	0	3.7	0
Fever	0	0	3.7	0
General fatigue	0	0	7.4	0
Dyspnea	8.0	0	3.7	0
Nausea	8.0	0	7.4	0
Vomiting	4.0	0	0	0
Appetite loss	0	4.0	0	0
Diarrhea	4.0	4.0	3.7	0
Stomatitis	0	0	3.7	0
Constipation	16.0	0	11.1	0
Atrial fibrillation	4.0	0	0	0
Eruption	12.0	4.0	7.4	0

G2 = Grade 2; G3 = grade 3. No grade 4 and 5 toxicities were observed. Adverse events were graded by the National Cancer Institute Common Toxicity Criteria version 2.0.

and to combine them with molecular targeted drugs. The CG combination was tested in various situations of NSCLC patients such as in an adjuvant setting, for the elderly and as a second line for selected patients, showing encouraging effects [13–15]. In addition, an acceptable toxicity and promising median overall survival was reported in the combination of CG and bevacizumab for Non-Sq NSCLC [16]. The management of carboplatin doublets is shown to be improved by introducing a clinical pathway [17].

We studied a 3-week CG regimen in which carboplatin is administered on day 8 and gemcitabine on days 1 and 8. In the present study, we compared two 3-week CG regimens, CG1 and CG8. The results show that the response rate, the primary endpoint of this study, is higher in CG1 than in CG8. The response rate in CG8 in our preceding phase II study described above was 22.6%, which was reproduced in this study. Since this study was conducted to select the better arm with regard to the response rate, one main conclusion of this study is that CG1 should be selected in subsequent studies.

In addition to the difference in response rate, our study also clarifies several useful differences between CG1 and CG8. In both hematologic and non-hematologic toxicities, CG8 is less toxic than CG1. Hematologic toxicities, especially thrombocytopenia, of CG8 are milder than those of CG1. Survival of the patients with CG8 is better than that of patients with CG1. Median survival time in the CG1 group is 348 days and is comparable to that of paclitaxel/carboplatin in the Four Arm Chemotherapy Study, a phase III study for advanced NSCLC performed in Japan [18]. Median survival time in the CG8 group is 455.5 days and is longer than that of CG1, although statistically not significant. The overall survival time of CG8 patients in our preceding phase II trial (454 days) was again reproduced. The difference in overall survival between CG1 and CG8 is hard to explain, because of the comparable dose intensity between the two arms and a better response rate in CG1. Reduced toxicity together with increased dose intensity of gemcitabine in CG8 may contribute to some extent to a possible prolonged survival. Increased dose intensity without deteriorating the patients' conditions may lead to prolonged survival and improvement in quality of life. Therefore, dose intensity could be selected as a primary endpoint in this study. The results show that dose intensities of both arms are comparable; the dose intensity of carboplatin is higher in CG1 and that of gemcitabine is higher in CG8. Progression-free survival time or overall survival time might have been a better endpoint. It may be possible to select tumor markers such as CYFRA21-1 or carcinoembryonic antigen as an endpoint in certain trials, because it is reported that they are valuable in evaluating chemotherapy in NSCLC [19].

CG was one of the least toxic platinum doublets. Recently, pemetrexed was introduced in the treatment of Non-Sq NSCLC. Pemetrexed/carboplatin produced comparable efficacy and showed statistically significant improvement in hematologic toxicities, when compared with CG1. Although pemetrexed/platinum is the first-choice regimen for Non-Sq NSCLC, CG is considered to be used for Sq NSCLC and even for Non-Sq NSCLC in an individual patient setting. In future chemotherapy for NSCLC, molecular targeted drugs will be used in combination with cytotoxic regimens. There are growing evidences showing that some molecular targeted drugs may require a suitable selection of cytotoxic regimens to exhibit cooperation [20–22]. Therefore, several kinds of platinum doublets, such as pemetrexed/platinum, paclitaxel/carboplatin and gemcitabine/platinum, should be tested in combination with new drugs. Our study may improve the usage of CG regimens, alone and in combination with molecular targeted drugs.

Conclusion

Whereas a standard 3-week schedule of CG (CG1) should be selected in subsequent studies because of a higher response rate, the new schedule (CG8) seems less toxic and shows a tendency of better survival.

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Preferential expression of potential markers for cancer stem cells in large cell neuroendocrine carcinoma of the lung. An FFPE proteomic study

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Abstract

Background: Large cell neuroendocrine carcinoma (LCNEC) of the lung, a subtype of large cell carcinoma (LCC), is characterized by neuroendocrine differentiation that small cell lung carcinoma (SCLC) shares. Pre-therapeutic histological distinction between LCNEC and SCLC has so far been problematic, leading to adverse clinical outcome. We started a project establishing protein targets characteristic of LCNEC with a proteomic method using formalin fixed paraffin-embedded (FFPE) tissues, which will help make diagnosis convincing.

Methods: Cancer cells were collected by laser microdissection from cancer foci in FFPE tissues of LCNEC (n = 4), SCLC (n = 5), and LCC (n = 5) with definite histological diagnosis. Proteins were extracted from the harvested sections, trypsin-digested, and subjected to HPLC/mass spectrometry. Proteins identified by database search were semi-quantified by spectral counting and statistically sorted by pair-wise G-statistics. The results were immunohistochemically verified using a total of 10 cases for each group to confirm proteomic results.

Results: A total of 1981 proteins identified from the three cancer groups were subjected to pair-wise G-test under $p < 0.05$ and specificity of a protein's expression to LCNEC was checked using a 3D plot with the coordinates comprising G-statistic values for every two group comparisons. We identified four protein candidates preferentially expressed in LCNEC compared with SCLC with convincingly low p-values: aldehyde dehydrogenase 1 family member A1 (AL1A1) ($p = 6.1 \times 10^{-4}$), aldo-keto reductase family 1 members C1 (AK1C1) ($p = 9.6 \times 10^{-10}$) and C3 (AK1C3) ($p = 3.9 \times 10^{-10}$) and CD44 antigen ($p = 0.021$). These p-values were confirmed by non-parametric exact inference tests. Interestingly, all these candidates would belong to cancer stem cell markers. Immunohistochmistry supported proteomic results.

Conclusions: These results suggest that candidate biomarkers of LCNEC were related to cancer stem cells and this proteomic approach via FFPE samples was effective to detect them.

Keywords: large cell neuroendocrine carcinoma, formalin-fixed paraffin embedded tissues, mass spectrometry, cancer stem cell markers

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. In Japan, annual deaths from lung cancer have been increasing and reached about 70,000 [2] and in USA reached 160,000 even with a recent decreasing trend [3]. Generally, lung cancer is divided into two histological subgroups, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). NSCLC mainly consists of adenocarcinoma (AC), squamous cell carcinoma (SC) and large cell carcinoma (LCC). AC and SC are differentiated with the features of normal cells but LCC is undifferentiated without such features. The prognosis of lung cancer depends on pathological stages and histological types; in NSCLC, AC is the best, while LCC the worst [4].

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Travis et al. [5] proposed a new subtype of LCC, named large cell neuroendocrine carcinoma (LCNEC) in 1991, and the World Health Organization finally adopted it for the revised pathological classification of lung cancer in 1999. LCNEC exhibits morphology similar to LCC but neuroendocrine differentiation like SCLC that could be judged by expression of at least one of three representative neuroendocrine proteins, CD56, synaptophysin (Syn) and chromogranin A (CGA). Among subtypes of LCC, the prognosis of LCNEC was poorer than others even if at early stages [6,7] like SCLC. However therapeutic strategies of LCNEC and SCLC differ from each other. The former needs surgery as the first choice but the latter chemotherapy. It is therefore important to distinguish LCNEC from SCLC definitely but common morphological growth patterns characteristic of neuroendocrine tumors sometimes hinder clear pathologic distinction between the two neuroendocrine cancers.

It follows that new biomarkers should be developed for definite diagnosis of those cancers, even if histopathology has long been the golden standard for diagnosis and determination of disease progression. Genomic and immunohistochemical analyses for such a purpose have been reported [8,9] but there have still been no biomarkers specific to LCNEC. Recent advancements in shotgun sequencing and quantitative mass spectrometry for protein analyses could make proteomics amenable to clinical biomarker discovery [10]. In addition, selective collection of target cells from formalin fixed paraffin embedded (FFPE) tissues by laser microdissection can permit to access to tissues of a variety of cancer types with definite diagnosis. We have used these methods for exploring stage-related proteins on non-metastatic lung AC by both global and multiple reaction monitoring (MRM) mass spectrometry-based proteomics [11,12]. In this study, we applied them to detect the potential protein markers characteristic of LCNEC by label-free semi-quantitative shotgun proteomics using spectral counting.

2. Materials and methods

2. 1. Sample Preparation for FFPE Tissue Specimens

Surgically removed lung tissues were fixed with a buffered formalin solution containing 10-15% methanol, and embedded by a conventional method. Archived paraffin blocks of formalin-fixed tissues obtained from four LCNEC cases, five LCC and five SCLC, which were retrieved with the approval from Ethical Committee of Tokyo Medical University Hospital and used with patients' consents. Patients' characteristics are listed in Table 1. Paraffin blocks were cut into 4 μm sections for diagnosis and 10 μm sections for proteomics. The 10 μm sections were stained with only haematoxylin. Three pathologists (M.N., H.O., and T.N.) independently made a diagnosis using the 4 μm sections stained with haematoxylin and eosin according to the WHO classification. LCNEC has its characteristic cancer cells with relatively larger cytoplasm, less fine chromatin and more distinct nucleoli than those of SCLC. The sections of patients diagnosed unequivocally were used in this study.

Table 1 Patients' Characteristics

Cancer groups	Patient No.	Gender	Age	TNM*	Staging
LCNEC	1	F	68	T1N0M0	IA
	2	M	73	T2N0M0	IB
	3	M	58	T1N1M0	IIA
	4	M	70	T2N0M0	IB
	5	M	76	T2N2M0	IIIA
	6	M	69	T3N3M0	IIIB
	7	M	64	T2N1M0	IIB
	8	M	60	T2N2M0	IIIA
	9	F	77	T1N0M0	IA
	10	M	69	T1N2M0	IIIA
SCLC	1	F	62	T2N0M0	IB
	2	M	77	T2N1M0	IIB
	3	M	57	T2N1M0	IIB
	4	M	76	T1N1M0	IIA
	5	M	64	T1N1M0	IIA
	6	F	70	T1N1M0	IIA
	7	M	69	T1N1M0	IIA
	8	M	77	T2N0M0	IB
	9	M	73	T1N0M0	IA
	10	M	73	T2N1M0	IIB
LCC	1	M	52	T2N1M0	IIB
	2	M	71	T1N0M0	IA
	3	F	57	T1N0M0	IA
	4	M	51	T4N2M0	IIIB
	5	M	72	T1N1M0	IIA
	6	M	67	T1N1M0	IIA
	7	M	67	T2N0M0	IB
	8	M	58	T1N0M0	IA
	9	M	67	T2N0M0	IB
	10	M	66	T1N0M0	IA

*Ref. [31].

2. 2. Immunohistochemical Staining

The neuroendocrine nature of tumors was confirmed with the three representative antibodies, monoclonal mouse anti CD56 antibody (Novocastra, Newcastle upon Tyne, U.K.), polyclonal rabbit anti CGA antibody (DAKO Japan, Kyoto, Japan) and monoclonal mouse anti SYN antibody (DAKO Japan, Kyoto, Japan). The staining of these antibodies was performed automatically on a Ventana Benchmark® XT (Ventana Japan, Tokyo, Japan). Expression of four proteomics-identifying proteins specific to LCNEC was tested with the following commercially available antibodies according to the manufacturer's protocols: monoclonal rabbit anti AL1A1 antibody (Abcom Japan, Tokyo, Japan),

polyclonal anti AK1C1 antibody (GeneTex, Irvine, CA, USA), monoclonal anti AK1C3 antibody (Sigma Japan, Tokyo, Japan) and monoclonal mouse anti CD44 antibody (Abcom Japan, Tokyo, Japan). Briefly, sections were incubated with xylene, rehydrated with graded ethanol solutions and incubated with methyl alcohol containing 3% hydrogen peroxide to remove endogenous peroxidase activity. After washing thoroughly with PBS, sections were incubated with adequately diluted primary antibodies and then with Histofine simple stain® (Nichirei Bioscience, Tokyo, Japan), and finally visualized with products of the peroxidase and diaminobenzidien reaction.

2. 3. Laser Capture and Protein Solubilization

Cancerous lesions were identified on serial sections of NSCLC tissues stained with hematoxylin-eosin (HE). For proteomic analysis, a 10 μm thick section prepared from the same tissue block was attached onto DIRECTOR™ slides (Expression Pathology, Rockville, MD, USA), deparaffinized twice with xylene for 5 min., rehydrated with graded ethanol solutions and distilled water and stained by only hematoxylin. Those slides were air-dried and subjected to laser microdissection with a Leica LMD6000 (Leica Micro-systems GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany). At least 30,000 cells (8.0mm^2) were collected directly into a 1.5mL low-binding plastic tube. Proteins were extracted and digested with trypsin using Liquid Tissue™ MS Protein Prep kits (Expression Pathology, Rockville, MD, USA) according to the manufacturer's protocol.

2. 4. Liquid Chromatography-Tandem Mass Spectrometry

We here adopted label-free semi-quantitation using spectral counting by liquid chromatography (LC)-tandem mass spectrometry (MS/MS) to a global proteomic analysis. The digested samples were analyzed in triplicates by LC-MS/MS using reversed-phase liquid chromatography (RP-LC) interfaced with a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via a *nano*-electrospray device as described in details previously [13]. Briefly, the RP-LC system consisted of a peptide Cap-Trap cartridge (0.5 × 2.0 mm) and a capillary separation column (an L-column Micro of 0.2 × 150 mm packed with reverse phase L-C18 gels of 3 μm in diameter and 12 nm pore size, (CERI, Tokyo, Japan)) connected an emitter tip (FortisTip of 20 μm ID and 150 μm OD with a perfluoropolymer-coated blunt end, OmniSeparo-TJ, Hyogo, Japan) to the outlet. An autosampler (HTC-PAL, CTC Analytics, Switzerland) loaded an aliquot of samples onto the trap, which then was washed with solvent A (98% distilled water with 2% acetonitrile and 0.1% formic acid) for concentrating peptides on the trap and desalting. Subsequently, the trap was connected in series to the separation column, and the whole columns were developed for 70 min. with a linear acetonitrile concentration gradient made from 5 to 40% solvent B (10% distilled water and 90% acetonitrile containing 0.1% formic acid) at the flow-rate of 1 μL/min. An LTQ was operated in the data-dependent MS/MS mode to automatically acquire up to three successive MS/MS scans in the centroid mode. The three most intense precursor ions for these MS/MS scans could be selected from a high-resolution MS spectrum (a survey scan) that an Orbitrap previously acquired during a predefined short time window in the profile mode at the resolution of 30 000 in the m/z range of 400 to 1600. The sets of acquired high-resolution MS and MS/MS spectra for peptides were converted to single data files and they were merged into Mascot generic format files for database searching.

2.5 Database Searching and Semi-quantification with Spectral Counting

Mascot software (version 2.1.1, Matrix Science, London, UK) was used for database search against Homo sapiens entries in the UniProtKB/Swiss-Prot database (Release 56.6, 20413 entries). Peptide mass tolerance was 10ppm, fragment mass tolerance 0.8Da, and up to two missed cleavages were allowed for errors in trypsin specificity. Carbamidomethylation of cysteines was taken as fixed modifications, and methionine oxidation and formylation of lysine, arginine and N-terminal amino acids as variable modifications. A p-value being < 0.05 was considered significant, and the score cutoff was 44. The lists of identified proteins were merged into a master file where the primary accession numbers and entry names from UniProtKB were used. The false positive rates for protein identification were estimated using a decoy database created by reversing the protein sequences in the original database; the estimated false positive rate of peptide matches was 0.45% under protein score threshold conditions ($p < 0.005$). Mascot search results were processed through Scaffold software (version 2.02.03, Proteome Software, Portland, OR) to semi-quantitatively analyze differential expression levels of proteins in LCNEC, LCC and SCLC by spectral counting as described [11]. The number of peptide MS/MS spectra with high confidence (Mascot ion score, $p < 0.005$) was used for calculating spectral counts. Fold changes of expressed proteins in the base 2 logarithmic scale (R_{SC}) were calculated using spectral counting as described [11]. Candidate proteins between two groups were chosen so that their R_{SC} satisfy >1 or <-1, which correspond to their fold changes >2 or <0.5. G-test was used for evaluating differential protein expression in pair-wise cancer groups [14]. In this study we mainly focus on LCNEC vs. SCLC comparison, but the other pairs were considered. The results are illustrated in a three-dimensional plot to judge whether a protein is specifically expressed in a given cancer group.

Although G-test does not require replicates, spectral counts for each protein from triplicates were pooled and used for G-statistic calculation using a two-way contingency table arranged in two rows for a target protein and any other proteins, and two columns for cancer groups on an Excel macro. Statistical significance should be $p < 0.05$. The Yates correction for continuity is applied to the 2 × 2 tables. The correction could enable us to handle the data containing small spectral counts including zero. Statisticians, however, showed that the results of G-test using a contingency table containing small counts are not so convincing because it is assumed that the G statistic asymptotically obey a χ^2 distribution with one degree of freedom. To validate the G-test results, we calculated exact *p*-values for some significant proteins without making any assumptions of statistical distribution based on the permutational distribution of the test statistic, i.e., Fisher's exact test and Mann-Whitney U test for the contingency tables using a R package.

3. Results

3. 1. Patient groups and pathological classification

To explore protein markers to distinguish LCNEC from SCLC, we investigated cancer cells prepared by laser microdissection from FFPE sections of LCNEC, SCLC, and LCC with a shotgun proteomic method. The LCNEC group consisted of four independent patients and other two groups consisted of five independent ones. For immunohistochemistry, we added more patients so as to amount to 10 patients for each group. Patients were divided into those cancer groups according to the WHO classification and by immunohistochemistry with antibodies raised against established neuroendocrine markers, CD56, CGA and Syn (Table 1 and Figure 1). All LCNEC and SCLC tissues used in this study are positively stained with at least one of these antibodies consistent with the neuroendocrine nature of those cancers. LCC tissues were not stained immunohistochemically except for 2 cases with faintly positive for Syn but histopathological differentiation from SC, AC and SCLC was required for its definite diagnosis. The patient profiles including the TNM pathological classification and staging are summarized in Table 1. There was no difference between the ages for each group ($p = 0.076$ by ANOVA, mean age ± SD: 68.4 ± 6.3 for LCNEC, 69.8 ± 6.8 for SCLC, and 62.8 ± 7.7 for LCC) and the number of male accounts for over 80% for all groups. The majority of patients remained at stages from IA to IIB and accordingly had the extent of the primary tumor (T1 and T2) and of regional lymph node involvement (N0 and N1) except for the most advanced stage IIIA or IIIB in a LCC patient (patient 4) and additional four patients of LCNEC for immunohistochemistry (patients 5, 6, 8, and 10). All patients had no distant metastasis (M0). All the patients but patient 5 (carboplatin + irinotecan) in LCNEC and patient 4 (carboplatin + pacritaxel) in LCC have not undergone pre-operative chemotherapy.

3. 2. LC-MS/MS protein identifications and semi-quantification by spectral counting

Trypsin-digests from laser-microdissected samples typically containing ~30,000 cells were analyzed in triplicate by LC-MS/MS as described in "Materials and Methods". Under the database search settings used, we identified significant proteins as follows: LCNEC contained a total of 1,124 proteins including 410 unique, 168 in the overlap only between LCNEC and SCLC, 93 in the overlap only between LCNEC and LCC, and 453 in the overlap among three groups; SCLC contained a total of 1,096 including 362 unique, 100 in the overlap only between SCLC and LCC and the overlapped proteins described above; LCC contained a total of 1,083 including 450 unique and the overlapped proteins described earlier. The spectral counts were calculated for these proteins and those from triplicate experiments were pooled, thereby improving the performance of G-test and decreasing false positive rates significantly [14]. There was no significant difference among the total spectral counts of each group ($p = 0.248$ by ANOVA; mean counts ± SD: 1916 ± 571 for LCNEC, 1879 ± 457 for SCLC, 2491 ± 645 for LCC). Next, the values of R_{sc} that is a measure of fold changes for protein expression levels were calculated as described in "Materials and Methods" using the spectral counts of these proteins. The pooled counts for each protein were also subjected to pair-wise G-test between cancer groups. Table 2 shows the identified proteins that are significantly up- or down-regulated in LCNEC compared with SCLC as judged by G test under $p < 0.05$. The proteins are listed in descending order of the R_{sc} values; the larger the R_{sc} value of a given protein, the greater its expression level in LCNEC compared with SCLC and vice versa. Representative proteins up-regulated in LCNEC were AL1A1, AK1C1, AK1C3, brain-type fatty acid-binding protein (FABP) and β-enolase. On the other hand, those in SCLC were brain acid soluble protein 1 (BASP), secretagogin (SEGN), fascin and neural cell adhesion molecule (CD56).

3. 3. Biomarker Candidates for LCNEC

To illustrate the specificity of protein expression toward LCNEC more clearly, we made a 3D scatter plot with an × axis indicating G-statistic values (*G* values) for LCNEC vs. LCC analysis, a y axis for LCC vs. SCLC, and a z axis for LCNEC vs. SCLC (Figure 2). When the spectral counts of a target protein are zero for both groups in question, it is hereafter defined as $G = 0$. The proteins expressed specifically to LCNEC will therefore be present in the region (x>3.84, z>3.84 corresponding to $p < 0.05$ each) on the x-z plane, those in SCLC in the region (y>3.84, z>3.84) on the y-z plane and those in LCC in the region (x>3.84, y>3.84)

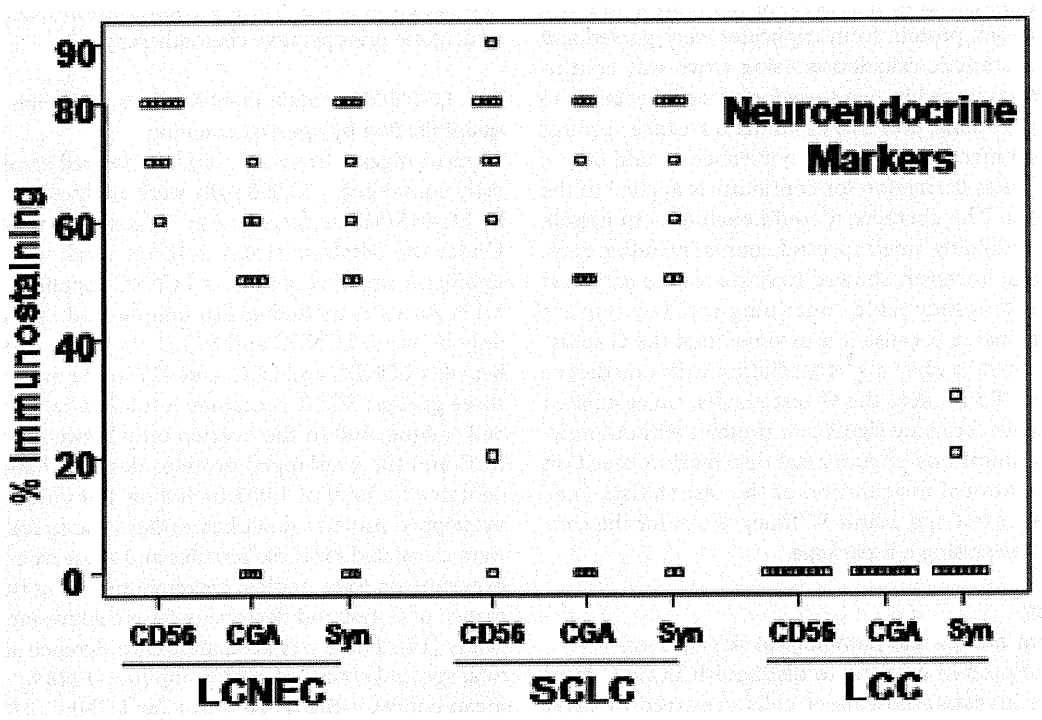

Figure 1 Immunohistochemistry with antibodies raised against established neuroendocrine markers, CD56, CGA, and Syn.

on the x-y plane. We used 1,918 proteins for this plotting. Close inspection of the 3D plot shows that AK1C3 at a point (40.8, 0, 39.1), AK1C1 at a point (39.0, 0, 37.4), AL1A1 at a point (8.75, 2.6×10^{-5}, 11.8) and CD44 antigen precursor (CD44) at a point (5.56, 0, 5.27) are very near or on the x-z plane with convincingly low p-values (3.9×10^{-10}, 9.6×10^{-10}, 6.1×10^{-4}, and 0.021, respectively) from LCNEC vs. SCLC comparisons and thus specific to LCNEC. Interestingly, AK1C1, AK1C3, AL1A1, and CD44 have been reported to be biomarkers of cancer stem cells (see Discussion). In Table 2 BASP and SEGN are significantly up-regulated in SCLC compared with LCNEC, which are indeed located on the y-z plane at the respective points (0, 32.2, 24.1) and (0, 21.5, 15.9), and specific to SCLC. Major vault protein (MVP) is at a point (23.8, 34.1, 0) on the x-y plane, indicating an LCC-specific protein. One of well known proteins related to SCLC, γ-enolase (ENOG) is detectable at a point (0.55, 7.23, 2.84) in the 3D G-statistic space which indicates that it is expressed significantly in SCLC compared to in LCC. The G-statistic is assumed to obey a $\chi 2$-distribution with one degree of freedom and the p-values based on G-values obtained with the contingency tables containing small counts should be handled with caution. Therefore we calculated exact p-values for the 2 × 2 tables with the non-parametric Fisher's exact test and Mann-Whitney U test. The results were fully consistent with those obtained with the G-test; the exact p-values for LCNEC vs. SCLC were 3.40×10^{-4} for AL1A1, 5.53×10^{-10} for AK1C1, 2.27×10^{-10} for AK1C3, and 0.012 for CD44. The G-test analyses of three cancer group pairs (LCNEC vs. SCLC, LCNEC vs. LCC, and LCC vs. SCLC) under $p < 0.05$ retrieved the respective 95, 186 and 237 proteins that showed significant changes in expression levels. These proteins were subjected to gene ontology (GO) analysis, highlighting their biological and molecular functions and cellular localization. As Figure 3 shows, the molecular functions and cellular localization of proteins preferentially expressed in the LCNEC vs. SCLC pair were quite different from those of the other pairs.

3. 4. Extended immunohistochemical validation of the proteomics results

From this proteomic study we identified AL1A1, AK1C1, AK1C3 and CD44 as biomarker candidates for LCNEC. The results were immunohistochemically verified using a total of 10 cases for each group. We assessed immunoreactivity with the percentage of immunopositive area and staining intensity compared to those of positive-control samples at the maximal cut-surface of tumors (Figure 4). All SCLC cases showed no immunoreactivity with AK1C1, AK1C3 and CD44 and the reactivity of all antibodies with LCNEC sections differed impressively from that of SCLC, supporting the proteomic results. Notably, nine cases of LCNEC including four used for the proteomic experiments

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

No	Entry name	Accession number	Proteins	G	P	Rsc	Spectral counts	
							LCNEC	SCLC
1	AK1C3	P42330	Aldo-keto reductase family 1 member C3	39.1	3.93E-10	4.91	25	0
2	AK1C1	Q04828	Aldo-keto reductase family 1 member C1	37.4	9.56E-10	4.86	24	0
3	FABP7	O15540	Fatty acid-binding protein, brain	21.9	2.89E-06	4.22	15	0
4	ENOB	P13929	Beta-enolase	22.2	2.50E-06	3.61	18	1
5	AL1A1	P00352	Retinal dehydrogenase 1	11.8	6.07E-04	3.55	9	0
6	4F2	P08195	4F2 cell-surface antigen heavy chain	11.8	6.07E-04	3.55	9	0
7	1C12	P30508	HLA class I histocompatibility antigen, Cw-12 alpha chain precursor	11.8	6.07E-04	3.55	9	0
8	TBA4A	P68366	Tubulin alpha-4A chain	11.8	6.07E-04	3.55	9	0
9	LG3BP	Q08380	Galectin-3-binding protein precursor	20.6	5.77E-06	3.54	17	1
10	1C03	P04222	HLA class I histocompatibility antigen, Cw-3 alpha chain precursor	10.1	1.48E-03	3.40	8	0
11	TKT	P29401	Transketolase	8.46	3.62E-03	3.24	7	0
12	VTNC	P04004	Vitronectin precursor	6.85	8.87E-03	3.05	6	0
13	G6PD	P11413	Glucose-6-phosphate 1-dehydrogenase	6.85	8.87E-03	3.05	6	0
14	PRDX4	Q13162	Peroxiredoxin-4	6.85	8.87E-03	3.05	6	0
15	VDAC1	P21796	Voltage-dependent anion-selective channel protein 1	6.85	8.87E-03	3.05	6	0
16	1B15	P30464	HLA class I histocompatibility antigen, B-15 alpha chain precursor	6.85	8.87E-03	3.05	6	0
17	VILI	P09327	Villin-1	6.85	8.87E-03	3.05	6	0
18	DESP	P15924	Desmoplakin	11.19	8.24E-04	2.96	11	1
19	AHSA1	O95433	Activator of 90 kDa heat shock protein ATPase homolog 1	5.27	2.18E-02	2.84	5	0
20	COPB	P53618	Coatomer subunit beta	5.27	2.18E-02	2.84	5	0
21	TMEDA	P49755	Transmembrane emp24 domain-containing protein 10 precursor	5.27	2.18E-02	2.84	5	0
22	CD44	P16070	CD44 antigen precursor	5.27	2.18E-02	2.84	5	0
23	COPA	P53621	Coatomer subunit alpha	5.27	2.18E-02	2.84	5	0
24	TBB4Q	Q99867	Putative tubulin beta-4q chain	5.27	2.18E-02	2.84	5	0
25	THIL	P24752	Acetyl-CoA acetyltransferase, mitochondrial precursor	5.27	2.18E-02	2.84	5	0
26	EFTU	P49411	Elongation factor Tu, mitochondrial precursor	14.88	1.14E-04	2.47	19	4
27	IDHP	P48735	Isocitrate dehydrogenase [NADP], mitochondrial precursor	13.55	2.32E-04	2.39	18	4
28	LRC47	Q8N1G4	Leucine-rich repeat-containing protein 47	5.41	2.01E-02	2.39	7	1
29	CO6A1	P12109	Collagen alpha-1(VI) chain precursor	4.08	4.34E-02	2.20	6	1
30	PSA	P55786	Puromycin-sensitive aminopeptidase	4.08	4.34E-02	2.20	6	1
31	IMB1	Q14974	Importin subunit beta-1	5.95	1.47E-02	2.17	9	2
32	PSA2	P25787	Proteasome subunit alpha type-2	4.72	2.99E-02	2.02	8	2
33	FAS	P49327	Fatty acid synthase	9.93	1.63E-03	1.93	18	6
34	A1AT	P01009	Alpha-1-antitrypsin precursor	4.05	4.41E-02	1.62	10	4
35	ROA1	P09651	Heterogeneous nuclear ribonucleoprotein A1	6.43	1.12E-02	1.58	16	7
36	FINC	P02751	Fibronectin precursor	8.01	4.64E-03	1.57	20	9
37	TRAP1	Q12931	Heat shock protein 75 kDa, mitochondrial precursor	6.26	1.24E-02	1.50	17	8
38	MYH14	Q7Z406	Myosin-14	6.26	1.24E-02	1.50	17	8
39	ANXA2	P07355	Annexin A2	5.46	1.94E-02	1.49	15	7
40	PHB2	Q99623	Prohibitin-2	4.67	3.07E-02	1.49	13	6
41	GSTP1	P09211	Glutathione S-transferase P	10.63	1.12E-03	1.38	32	17
42	PDIA1	P07237	Protein disulfide-isomerase precursor	8.96	2.76E-03	1.33	29	16
43	1433G	P61981	14-3-3 protein gamma	8.11	4.40E-03	1.28	28	16
44	ACTN4	O43707	Alpha-actinin-4	8.82	2.98E-03	1.26	31	18
45	PCBP2	Q15366	Poly(rC)-binding protein 2	5.03	2.49E-02	1.16	21	13
46	TPIS	P60174	Triosephosphate isomerase	6.45	1.11E-02	1.12	28	18
47	TRFE	P02787	Serotransferrin precursor	7.17	7.41E-03	1.12	31	20
48	ARF1	P84077	ADP-ribosylation factor 1	5.00	2.53E-02	1.09	23	15
49	PCBP1	Q15365	Poly(rC)-binding protein 1	4.31	3.79E-02	1.01	23	16