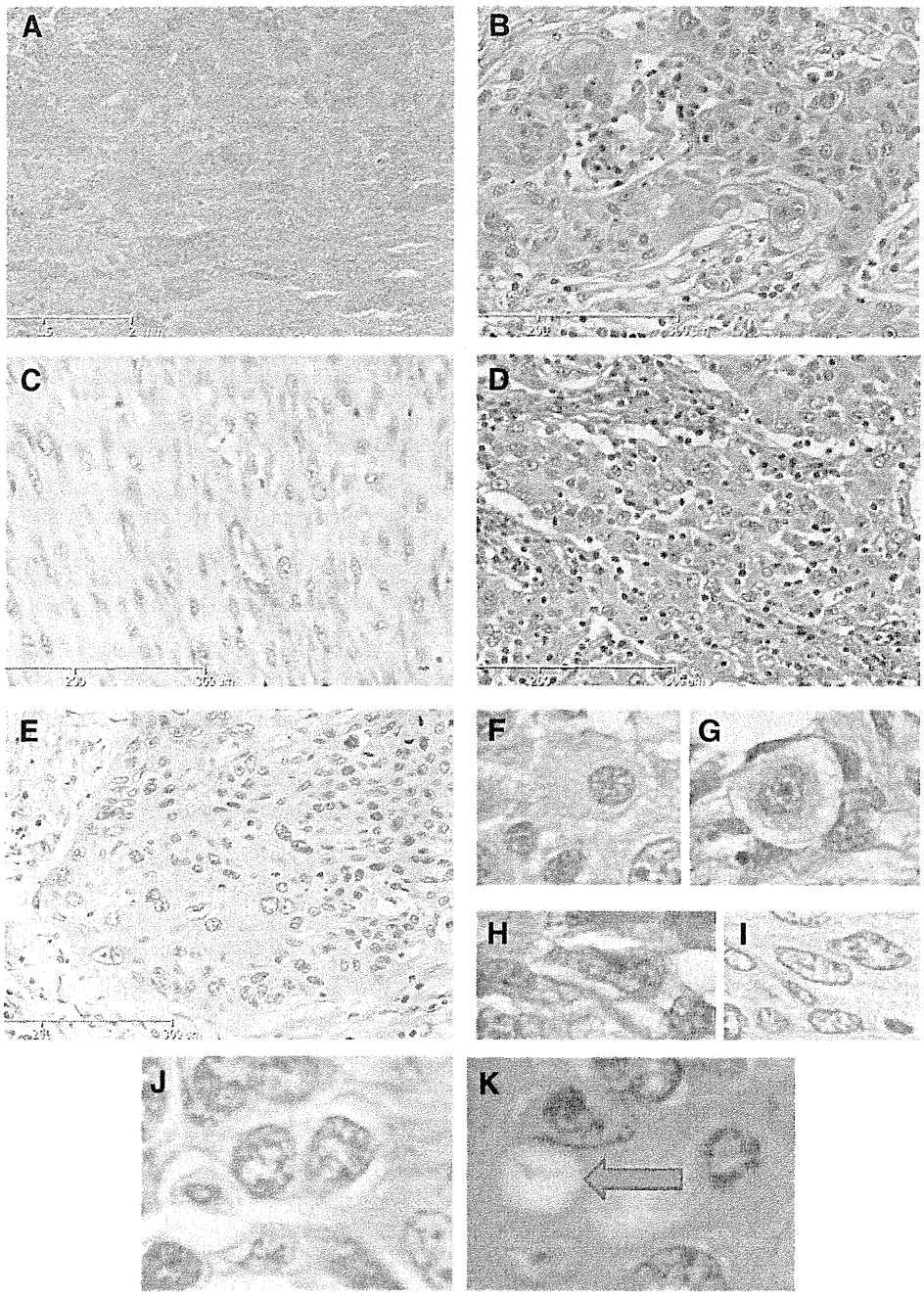

FIGURE 2. Example of consensus squamous cell carcinoma. *A*, Overview, ×20 (*B*), example of spindle cells in diffuse architecture (no nests or fields) (*C*), consensus score for lymphocytic infiltrate between the tumor cells (*D*), For palisading, no consensus was obtained: 25% of the pathologists called this palisading (*E*), detailed images with around the centered cell in at least part of the perimeter features of intercellular bridges (*F, G*), detailed images with three examples of intercellular gaps (called by most pathologists) (*H–J*), example of intracytoplasmic vacuole (arrow, consensus by all the pathologists) (*K*).

Basaloid carcinoma was described in 1992. The cardinal histopathologic features distinguishing this tumor from the other NSCLCs are a lobular growth pattern of small cells with moderately hyperchromatic nuclei without prominent nucleoli, and with scant cytoplasm, a high mitotic rate, and peripheral palisading.[20] This pattern could be present in a pure form or mixed with SqCC and was associated with a poor prognosis in stage I cases when compared to other NSCLCs.[21] When the prognosis of basaloid carcinomas is compared to poorly differentiated SqCCs, however, no differences were found, although the case numbers in these studies were relatively small.[22,23] In many instances, the differential diagnosis includes SCLC small-cell lung cancer and large cell neuroendocrine carcinoma (LCNEC).[24–27] Additional staining showing positive 34βE12 and p63 may suggest a diagnosis of basaloid SqCC. Taking the additional information from this study into account, arguments exist to consider basaloid carcinoma as a variant of SqCC. In this instance, IHC is superimposable with this interpretation.

Limitations of this study are that (1) the reader had only one section in images to examine, whereas the submission diagnosis was based on all the sections on glass slides

available; (2) IHC outcome was used and not the reproducibility of IHC interpretation; (3) images were scanned at ×20 magnification, making interpretation of subtle morphologic features at ×40 less optimal.

In conclusion, the histologic criteria that may be used in the differential diagnosis of poorly differentiated lung cancer need to be more precisely refined. Furthermore, additional stains improve the reproducibility of histological diagnosis of SqCC and AdC, uncovering information that is not present in regular H&E-stained slides.

REFERENCES

1. Travis W, Brambilla E, Muller-Hermelink H (Eds.), WHO Classification of Tumours, Pathology and Genetics: Tumors of the Lung, Pleura Thymus and Heart. Lyon: IARC Press LYON, 2004.
2. Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma. *J Thorac Oncol* 2011;6:244–285.
3. Thunnissen E, Beasley MB, Borczuk AC, et al. Reproducibility of histopathological subtypes and invasion in pulmonary adenocarcinoma. An international interobserver study. *Mod Pathol* 2012;25:1574–1583.
4. Hanai A, Whittaker JS, Tateishi R, Sobin LH, Benn RT, Muir CS. Concordance of histological classification of lung cancer with special reference to adenocarcinoma in Osaka, Japan, and the North-West Region of England. *Int J Cancer* 1987;39:6–9.
5. Feinstein AR, Gelfman NA, Yesner R. Observer variability in the histopathologic diagnosis of lung cancer. *Am Rev Respir Dis* 1970;101:671–684.
6. Colby TV, Tazelaar HD, Travis WD, Bergstralh EJ, Jett JR. Pathologic review of the Mayo Lung Project cancers [corrected]. Is there a case for misdiagnosis or overdiagnosis of lung carcinoma in the screened group? *Cancer* 2002;95:2361–2365.
7. Grilley-Olson JE, Hayes DN, et al. Validation of interobserver agreement in lung cancer assessment hematoxylin-eosin diagnostic reproducibility for non-small cell lung cancer—the 2004 World Health Organization Classification and Therapeutically Relevant Subsets. *Arch Pathol Lab Med* 2013;137:32–40.
8. Thunnissen E, Boers E, Heideman DA, et al. Correlation of immunohistochemical staining p63 and TTF-1 with EGFR and K-ras mutational spectrum and diagnostic reproducibility in non small cell lung carcinoma. *Virchows Arch* 2012;461:629–638.
9. Scagliotti GV, Parikh P, von Pawel J, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol* 2008;26:3543–3551.
10. Roggli VL, Vollmer RT, Greenberg SD, McGavran MH, Spjut HJ, Yesner R. Lung cancer heterogeneity: a blinded and randomized study of 100 consecutive cases. *Hum Pathol* 1985;16:569–579.
11. Stanley KE, Matthews MJ. Analysis of a pathology review of patients with lung tumors. *J Natl Cancer Inst* 1981;66:989–992.
12. Tsuta K, Tanabe Y, Yoshida A, et al. Utility of 10 immunohistochemical markers including novel markers (desmocollin-3, glypican 3, S100A2, S100A7, and Sox-2) for differential diagnosis of squamous cell carcinoma from adenocarcinoma of the Lung. *J Thorac Oncol* 2011;6:1190–1199.
13. Rossi G, Mengoli MC, Cavazza A, et al. Large cell carcinoma of the lung: clinically oriented classification integrating immunohistochemistry and molecular biology. *Virchows Arch* 2014;464:61–68.
14. Rossi G, Tiseo M, Cavazza A, Colby T V. Is immunohistochemistry always required to diagnose lung cancer? *Adv Anat Pathol* 2013;20:327–333.
15. Hwang DH, Szeto DP, Perry AS, et al. Pulmonary large cell carcinoma lacking squamous differentiation is clinicopathologically indistinguishable from solid-subtype adenocarcinoma. *Arch Pathol Lab Med* 2014;138:626–635.
16. Monica V, Ceppi P, Righi L, et al. Desmocollin-3: a new marker of squamous differentiation in undifferentiated large-cell carcinoma of the lung. *Mod Pathol* 2009;22:709–717.
17. Pardo J, Martinez-Penuela AM, Sola JJ, et al. Large cell carcinoma of the lung: an endangered species? *Appl Immunohistochem Mol Morphol* 2009;17:383–392.
18. Yesner R, Gerstl B, Auerbach O. Application of the World Health Organization Classification of Lung Carcinoma to Biopsy Material. *Ann Thorac Surg* 1965;10:33–49.
19. Rekhtman N, Paik PK, Arcila ME, et al. Clarifying the spectrum of driver oncogene mutations in biomarker-verified squamous carcinoma of lung: lack of EGFR/KRAS and presence of PIK3CA/AKT1 mutations. *Clin Cancer Res* 2012;18:1167–1176.
20. Brambilla E, Moro D, Veale D, et al. Basal Cell (Basaloid) Carcinoma of the Lung : A new Morphologic and Phenotypic entity With Separate Prognostic Significance. *Hum Pathol* 1992;23:993–1003.
21. Moro-Sibilot D, Lantuejoul S, et al. Lung carcinomas with a basaloid pattern: a study of 90 cases focusing on their poor prognosis. *Eur Respir J* 2008;31:854–859.
22. Wang LC, Wang L, Kwauk S, et al. Analysis on the clinical features of 22 basaloid squamous cell carcinoma of the lung. *J Cardiothorac Surg* 2011;6:10.
23. Kim DJ, Kim KD, Shin DH, Ro JY, Chung KY. Basaloid carcinoma of the lung: a really dismal histologic variant? *Ann Thorac Surg* 2003;76:1833–1837.
24. Maleki Z. Diagnostic issues with cytopathologic interpretation of lung neoplasms displaying high-grade basaloid or neuroendocrine morphology. *Diagn Cytopathol* 2011;39:159–167.
25. Crapanzano JP, Loukeris K, Borczuk AC, Saqi A. Cytological, histological, and immunohistochemical findings of pulmonary carcinomas with basaloid features. *Diagn Cytopathol* 2010;39:92–100.
26. Kim MJ, Ha SY, Kim NR, Cho HY, Chung DH, Kim GY. Aspiration cytology features of pulmonary basaloid carcinoma. *Cytopathology* 2009;20:336–339.
27. Yamada S, Noguchi H, Nabeshima A, et al. Basaloid carcinoma of the lung associated with central cavitation: a unique surgical case focusing on cytological and immunohistochemical findings. *Diagn Pathol* 2013;7:175.

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Adjuvant Chemotherapy in Patients with Completely Resected Small Cell Lung Cancer: A Retrospective Analysis of 26 Consecutive Cases

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Objective: Several clinical studies have demonstrated the efficacy and safety of adjuvant chemotherapy in patients with completely resected small cell lung cancer for a selected limited stage. However, it is unclear whether adjuvant chemotherapy is feasible in clinical practice. The objective of this study was to analyze the efficacy and safety of adjuvant chemotherapy for small cell lung cancer patients retrospectively in clinical practice.
Methods: From January 2002 to March 2012, 56 small cell lung cancer patients underwent surgery as initial therapy in our institute. Of these, 26 patients received adjuvant chemotherapy. The clinical data of patients who received adjuvant chemotherapy were retrospectively analyzed.
Results: The chemotherapy regimens were cisplatin and irinotecan in 16 patients, cisplatin and etoposide in 1 and carboplatin and etoposide in 9. Median follow-up time was 44.8 months. Nineteen (73%) patients received the full course of chemotherapy. Median recurrence-free survival was 21.4 months. Median survival time was not reached. There was no treatment-related death.
Conclusion: Adjuvant chemotherapy may be generally safe and efficacious in selected small cell lung cancer patients.

Key words: small cell lung cancer – surgery – adjuvant chemotherapy

INTRODUCTION

Small cell lung cancer (SCLC) accounts for approximately 15% of lung cancers. It is a virulent, rapidly growing, early metastasizing and invasive cancer. At diagnosis, approximately 90% of patients with SCLC already have regional or distant spread (1). Furthermore, it is difficult to diagnose SCLC presenting as a solitary small nodule of the lung by transbronchial lung biopsy. As a result, SCLC presenting as a solitary small nodule is often diagnosed at the time of therapeutic surgical resection. In these cases, we commonly administer additional chemotherapy after surgery in clinical practice to control micro metastases. A previous clinical study, case series and a meta-analysis showed that adjuvant chemotherapy might be feasible and reduce the risk of recurrence in SCLC patients (2–4). In addition, Tsuchiya et al. (5) reported that surgical resection followed by cisplatin and etoposide chemotherapy was feasible. The European Society for Medical Oncology (ESMO) and American College of Chest Physicians (ACCP) guidelines recommend adjuvant chemotherapy for SCLC patients. However, it was unclear that adjuvant

chemotherapy for SCLC patients was efficacy and safety in clinical practice. Therefore, the efficacy and safety of adjuvant chemotherapy for SCLC patients were retrospectively analyzed.

PATIENTS AND METHODS

The current study included 56 consecutive patients with histologically proven SCLC who underwent complete resection at the National Cancer Center Hospital (NCCH) from January 2002 to March 2012. The medical records of SCLC patients who received adjuvant chemotherapy were retrospectively reviewed. Patients who had post-operative recurrence before starting adjuvant chemotherapy, patients who had difficulty with adjuvant chemotherapy due to complications, and patients who refused were excluded. No patients had received any treatment such as chemotherapy or irradiation before surgery. Histological diagnoses and tumor grades were determined in accordance with TNM staging (seventh edition) (6). The following data were extracted: (i) patients' characteristics: age, sex and Eastern Cooperative Oncology Group Performance Status (ECOG PS) at the start of adjuvant chemotherapy, clinical stage before surgery, pathological stage after surgery and histological diagnosis before and after surgery; (ii) type of chemotherapeutic agents administered, dose, treatment cycle, relative dose intensity and toxicity; and (iii) patterns of recurrence, recurrence-free survival time (RFS) and overall survival time (OS) data. All the patients gave their written informed consent to analyze their medical records after treatments. This study was approved by the Institutional Review Board of NCCH.

Treatment Schedule

The chemotherapy regimens were cisplatin and irinotecan (IP), cisplatin and etoposide (EP) and carboplatin and etoposide (CE). The doses of the chemotherapeutic agents were: cisplatin (60 mg/m^2 on Day 1) and irinotecan (60 mg/m^2 on Days 1, 8 and 15) repeated every 4 weeks; cisplatin (80 mg/m^2 on Day 1) and etoposide (100 mg/m^2 on Days 1–3) repeated every 3 weeks; and carboplatin (AUC = 5 on Day 1) and etoposide (80 mg/m^2 on Days 1–3) repeated every 3 weeks. All regimens consisted of a total of four cycles. The efficacy and safety of each regimen has been established in previous clinical trials (5,7,8).

Assessment and Analysis

Safety and tolerability were assessed during the adjuvant chemotherapy. Adverse events were graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. RFS and OS were measured from the date of surgery until recurrence and death or the final day of the follow-up period, and median survival was calculated using the Kaplan–Meier method. STATA version 12 (StataCorp LP, College Station, TX, USA) was used for all analyses.

Table 1. Patients' characteristics

Characteristic	*N*
Twenty-six patients received adjuvant chemotherapy	
Total	26
Sex	
Male/female	19/7
Age	
Median (range)	67 (46–84)
ECOG PS	
0/1	21/5
Clinical stage	
I (T1N0M0/T2aN0M0)	22 (17/5)
II (T1N1M0/T2aN1M0/T3N0M0)	4 (1/2/1)
III 0	
Pathological stage	
I (T1N0M0/T2aN0M0)	10 (6/4)
II (T2bN0M0/T1N1M0/T2N1M0/T3N0M0)	9 (1/2/4/2)
III (T1N2M0/T2N2M0/T3N2M0)	7 (4/2/1)
Pathological histology	
Small cell carcinoma	18
Combined small cell carcinoma	
With adenocarcinoma	4
With large cell carcinoma	4
Thirty patients received surgery alone	
Total	30
Sex	
Male/female	25/5
Age	
Median (range)	71 (57–89)
ECOG PS	
0/1	13/17
Clinical stage	
I (T1N0M0/T2aN0M0)	25 (21/4)
II (T1N1M0/T2N1M0)	4 (2/2)
III (T3N1M0)	1 (1)
Pathological stage	
I (T1N0M0/T2aN0M0)	18 (15/3)
II (T1N1M0/T2N1M0/T3N0M0)	7 (3/2/2)
III (T1N2M0/T2N2M0/T3N2M0/T4N2M0/T3N3M0)	5 (1/1/1/1 /1)
Pathological histology	
Small cell carcinoma	19
Combined small cell carcinoma	
With adenocarcinoma	4
With large cell carcinoma	4
With squamous cell carcinoma	3

ECOG PS, Eastern Cooperative Oncology Group Performance Status; *N*, number of patients.

RESULTS

Patient Characteristics

A total of 56 consecutive patients with SCLC were sampled from the hospital-based registry of the NCCH between January 2002 and March 2012. The characteristics of the patients are listed in Table 1. All patients underwent surgery as initial treatment. The surgical procedures were pulmonary lobectomy in 55 patients and partial resection in one patient. Thirty patients were excluded for reasons such as death not relevant to surgery ($n = 1$), early post-operative recurrence ($n = 2$), thoracic empyema after surgery to need antibiotics for long periods ($n = 2$), severe complications ($n = 4$) and poor general condition including old age ($n = 5$) (Fig. 1). As a result, 26 patients who received adjuvant chemotherapy were reviewed in this study.

Discrepancy Between Clinical and Pathological Histology Findings and Stages

Only 9 patients had a confirmed diagnosis of SCLC and 13 patients did not have a confirmed diagnosis before surgery. On the other hand, in four patients, the confirmed diagnosis was changed to SCLC. Their pre-operative diagnoses included one adenocarcinoma, one squamous cell carcinoma, one large cell carcinoma and one carcinoma not otherwise specified, respectively. As a consequence of surgery, combined SCLC types with adenocarcinoma or squamous cell carcinoma were found in 8 (30.8%) patients. Twenty-two patients had pre-operative clinical Stage I disease and four had Stage II disease. However, post-operative pathological Stage I, II and III disease was found in 10, 9 and 7 patients, respectively (Table 1).

Chemotherapy Regimens

The chemotherapy regimen was selected by each physician. Sixteen patients received IP, one received EP and nine received CE (Table 2). The median age of the patients who received IP was 65 years (range, 47–72 years), while that of patients who received CE was 75 years (range, 62–84 years). Most patients who were 70 years of age or older received CE (88.9%).

Treatment Delivery and Relative Dose Intensity

The median duration from surgery to starting chemotherapy was 51 days (range, 26–78 days). Table 3 shows treatment delivery for each regimen. Nineteen (73%) patients received four cycles of chemotherapy. Seven (27%) patients did not

Table 2. Regimen selected

	Number of patients	Median age (range)	ECOG PS 0/1 (*N*)
IP	16	65 (47–72)	10/6
EP	1	46	1/0
CE	9	75 (62–84)	4/5

IP, cisplatin and irinotecan; EP, cisplatin and etoposide; CE, carboplatin and etoposide.

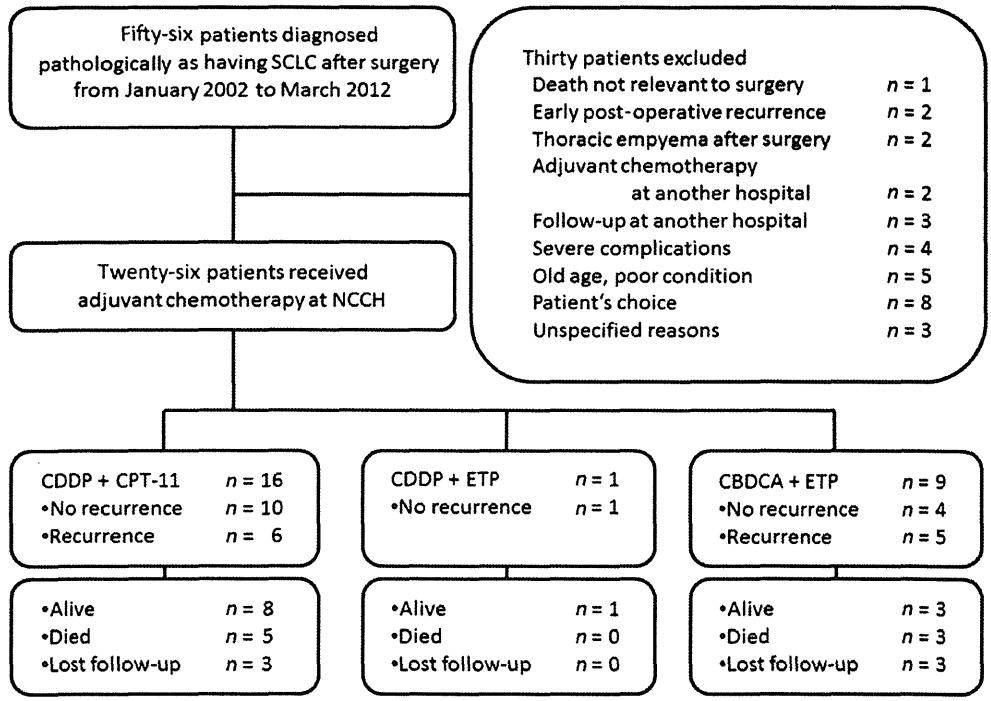

Figure 1. Follow-up of the study patients by treatment group after surgery.

complete the initially planned chemotherapy because of adverse events (AE). The relative dose intensity was 83.6% in IP, 87.5% in EP, and 86.8% in CE.

Safety Analysis

Chemotherapy-related toxicity is shown in Table 4. Grade 4 AEs were found in 14 (53.8%) patients: neutropenia in 11 patients, thrombocytopenia in 2 patients and febrile neutropenia in 1 patient. Adjuvant chemotherapy for completely resected SCLC patients was feasible. All AEs were manageable, and there was no treatment-related death. We had to stop or change chemotherapy regimens due to AEs in four patients received IP and three patients received CE. In IP, two patients were changed to EP due to hepatic toxicity, one patient was changed to CE due to kidney failure and one patient could not continue to receive chemotherapy due to brain bleeding. In CE, all three patients discontinued chemotherapy due to fatigue and allergy. These three patients were over the age of 70 years (Table 3).

Efficacy Analysis

Of the 26 patients, 18 (69.2%) were still alive after the median follow-up of 44.8 months (range, 2.8–78.1 months). The median RFS of all patients was 21.4 months (95% CI: 14.6–41.3 months); the median RFS was 17.8 months (95% CI: 12.8–46.5 months) with IP and 23.0 months with CE (95% CI: 10.2–61.9 months) (Fig. 2A). The median survival time of all patients could not be calculated due to the insufficient follow-up time. The estimated 3-year and 5-year survivals were 68.9% (95% CI: 42.3–84.6%) and 51.7% (95% CI: 24.0–73.2%), respectively (Fig. 2B). On the other hand, the estimated 3-year and 5-year survivals of 30 patients received surgery alone were 60.5% (95% CI: 39.9–76.0%) and 45.4% (95% CI: 25.0–63.8%), respectively.

Table 3. Treatment delivery

Number of treatment cycles	IP ($N = 16$)	EP ($N = 1$)	CE ($N = 9$)	Total ($N = 26$)
4	12 (75%)	1 (100%)	6 (67%)	19 (73%)
3	–	–	1 (11%)	1 (4%)
2	–	–	1 (11%)	1 (4%)
1	4 (25%)	–	1 (11%)	5 (19%)

Table 4. Chemotherapy-related toxicity by CTC-AE ver. 4.0

	Grade				
Toxicity	1	2	3	4	3/4
Anemia	10	3	2	0	2 (8)
Neutropenia	1	0	3	11	14 (54)
Febrile neutropenia	0	0	2	1	3 (12)
Thrombocytopenia	1	3	3	2	5 (19)
Nausea	12	3	1	0	1 (4)
Appetite loss	11	5	0	0	0 (0)
Diarrhea	7	5	1	0	1 (4)
Fatigue	8	2	1	0	1 (4)
Hepatic dysfunction	1	0	2	0	2 (8)
Renal failure	1	1	0	0	0 (0)

Values are N (%).

Patterns of Recurrence

Recurrence was confirmed in 10 (38.5%) patients, and the initial recurrence site was mediastinal lymph nodes in three patients, lung in three, bone in three and abdominal lymph node in one. Recurrence was found in two patients with pathological Stage I, four patients with Stage II, and four patients with Stage IIIA.

DISCUSSION

Although the standard treatment for most cases of limited SCLC is considered to be chemoradiotherapy, clinical T1 and T2 SCLC without evidence of lymph node involvement (N0) can be considered for surgical resection. Previous reports suggested that these selected patients might benefit from surgery expecting radical cure (9–11). In addition, combination surgery and adjuvant chemotherapy or post-operative irradiation has a 5-year survival of approximately 40–70% (2–5). However, it is difficult to diagnose T1 and T2 SCLC presenting as a solitary pulmonary nodule prior to surgery despite development of less invasive diagnostic methods such as transbronchial lung biopsy, endobronchial ultrasonography and CT-guided lung biopsy (12). As a result, SCLC presenting as a solitary pulmonary nodule is often diagnosed at the time of therapeutic resection. In the present analysis, 13 patients underwent surgery with uncertain pathological diagnoses. Furthermore, four patients had a diagnosis of NSCLC before surgery. According to previous reports, approximately 5–10% of patients diagnosed with SCLC will have other pathologies such as adenocarcinoma or squamous cell carcinoma within the surgically resected specimens (13,14). As a consequence of surgery, combined SCLC types with adenocarcinoma or squamous cell carcinoma were found in 8 (30.8%) patients. We have no defined treatment strategy for combined SCLC (containing any other NSCLC component). However, it has been reported that there is no difference in the prognosis between SCLC and combined SCLC (15). In our perspective, surgery would be the best treatment choice for early stage combined SCLC.

There have been no Phase III trials of adjuvant chemotherapy for SCLC. A previous clinical study, a case series, and a meta-analysis showed that adjuvant chemotherapy including cisplatin may be feasible and reduce the risk of recurrence in SCLC patients (2–4). The feasibility of EP after surgical resection has

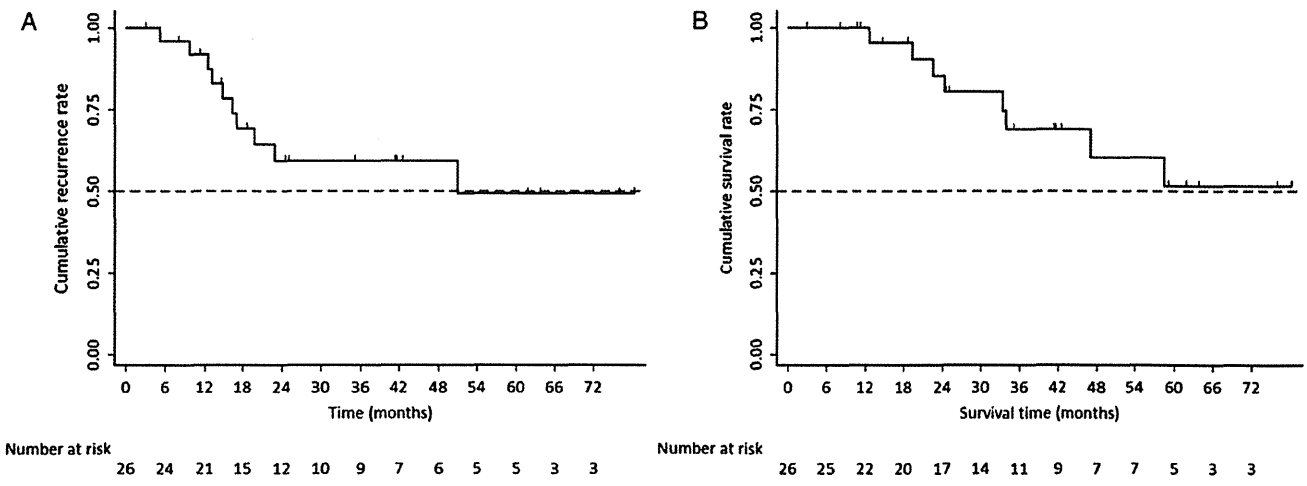

Figure 2. (A) Recurrence-free survival among the study patients. Kaplan–Meier curves for recurrence-free survival are shown for the recurrence-free survival population. (B) Overall survival among the study patients. Kaplan–Meier curves for overall survival are shown for the overall survival population.

been reported from Japan (2,5). Therefore, it remains unclear which regimen is appropriate. According to previous clinical trials of extensive disease-SCLC (7,8), EP, IP and CE were selected for adjuvant chemotherapy regimens. In the present analysis, the choice of regimen was left to the physician by reference to previous clinical trials (5,7,8). Regarding efficacy, we consider that IP and CE were not apparently inferior to EP in a previous Phase II study (JCOG 9101) in which the estimated 3-year and 5-year survivals were 61 and 57%, respectively.

The CE regimen has been used in elderly or poor-risk patients with extensive disease-SCLC (8). In the present analysis, CE had acceptable toxicities and reproducible efficacy in this population. In the period of the present analysis, surgery was performed as initial therapy for 56 SCLC patients at the NCCH. Of these, 30 patients could not receive adjuvant chemotherapy for any reason. Therefore, those who received surgery and adjuvant chemotherapy in this study were highly selected. Thirty patients received surgery alone tended to be in higher median age and in poor PS compared with these received adjuvant chemotherapy. But, we could not show clearly-defined cut-off line of adjuvant chemotherapy. It is the limitation of this retrospective study.

A phase III trial of EP versus IP for adjuvant chemotherapy (UMIN 000010298) is now ongoing in patients diagnosed with high-grade pulmonary neuroendocrine carcinoma (large cell neuroendocrine carcinoma and small cell lung cancer) by the Japan Clinical Oncology Group (JCOG).

Adjuvant chemotherapy of selected SCLC patients may be generally safe and efficacious. Further studies should be considered to evaluate the therapeutic possibility of adjuvant chemotherapy in SCLC patients.

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Conflict of interest statement

None declared.

References

1. Sher T, Dy GK, Adjei AA. Small cell lung cancer. *Mayo Clinic Proc* 2008;83:355–67.
2. Wada H, Yokomise H, Tanaka F, et al. Surgical treatment of small cell carcinoma of the lung: advantage of preoperative chemotherapy. *Lung Cancer* 1995;13:45–56.
3. Karrer K, Shields TW, Denck H, Hrabar B, Vogt-Moykopf I, Salzer GM. The importance of surgical and multimodality treatment for small cell bronchial carcinoma. *J Thorac Cardiovasc Surg* 1989;97:168–76.
4. Brock MV, Hooker CM, Syphard JE, et al. Surgical resection of limited disease small cell lung cancer in the new era of platinum chemotherapy: its time has come. *J Thorac Cardiovasc Surg* 2005;129:64–72.
5. Tsuchiya R, Suzuki K, Ichinose Y, et al. Phase II trial of postoperative adjuvant cisplatin and etoposide in patients with completely resected stage I–IIIa small cell lung cancer: the Japan Clinical Oncology Lung Cancer Study Group Trial (JCOG9101). *J Thorac Cardiovasc Surg* 2005;129:977–83.
6. Greene FL, Sobin LH. A worldwide approach to the TNM staging system: collaborative efforts of the AJCC and UICC. *J Surg Oncol* 2009;99:269–72.
7. Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *New Engl J Med* 2002;346:85–91.

8. Okamoto H, Watanabe K, Kunikane H, et al. Randomised phase III trial of carboplatin plus etoposide vs split doses of cisplatin plus etoposide in elderly or poor-risk patients with extensive disease small-cell lung cancer: JCOG 9702. *Br J Cancer* 2007;97:162–9.
9. Namikawa S, Den T, Kimura M, Kusagawa M. The role of surgical resection and the effects of neo-adjuvant therapy in the management of small cell lung cancer. *Surg Today* 1994;24:342–6.
10. Niiranen A. Long-term survival in small cell carcinoma of the lung. *Eur J Cancer Clin Oncol* 1988;24:749–52.
11. Maassen W, Greschuchna D. Small cell carcinoma of the lung—to operate or not? Surgical experience and results. *Thorac Cardiovasc Surg* 1986;34:71–6.
12. Quoix E, Fraser R, Wolkove N, Finkelstein H, Kreisman H. Small cell lung cancer presenting as a solitary pulmonary nodule. *Cancer* 1990;66:577–82.
13. Shepherd FA, Ginsberg RJ, Feld R, Evans WK, Johansen E. Surgical treatment for limited small-cell lung cancer. The University of Toronto Lung Oncology Group experience. *J Thorac Cardiovasc Surg* 1991;101: 385–93.
14. Mangum MD, Greco FA, Hainsworth JD, Hande KR, Johnson DH. Combined small-cell and non-small-cell lung cancer. *J Clin Oncol* 1989;7:607–12.
15. Nicholson SA, Beasley MB, Brambilla E, et al. Small cell lung carcinoma (SCLC): a clinicopathologic study of 100 cases with surgical specimens. *Am J Surg Pathol* 2002;26:1184–97.

OPEN

Therapeutic Priority of the PI3K/AKT/mTOR Pathway in Small Cell Lung Cancers as Revealed by a Comprehensive Genomic Analysis

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Introduction: The information regarding therapeutically relevant genomic alterations in small cell lung cancer (SCLC) is not well developed. We analyzed the SCLC genome using an integrative approach to stratify the targetable alterations.

Methods: We performed whole exon sequencing (n = 51) and copy number analysis (n =47) on surgically resected tumors and matched normal tissue samples from treatment-naive Japanese SCLC patients.

Results: The demographics of the 51 patients included in this study were as follows: median age, 67 years (range, 42–86 years); female, 9 (18%); history of smoking, 50 (98%); and pathological stage I/II/III/IV, 28/13/9/1, respectively. The average number of nonsynonymous mutations was 209 (range, 41–639; standard deviation, 130). We repeatedly confirmed the high prevalence of inactivating mutations in *TP53* and *RB1*, and the amplification of *MYC* family members. In addition, genetic alterations in the PI3K/AKT/mTOR pathway were detected in 36% of the tumors: *PIK3CA*, 6%; *PTEN*, 4%; *AKT2*, 9%; *AKT3*, 4%; *RICTOR*, 9%; and *mTOR*, 4%. Furthermore, the individual changes in this pathway were mutually exclusive. Importantly, the SCLC cells harboring active *PIK3CA* mutations were potentially targetable with currently available PI3K inhibitors.

Conclusions: The PI3K/AKT/mTOR pathway is distinguishable in SCLC genomic alterations. Therefore, a sequencing-based comprehensive analysis could stratify SCLC patients by potential therapeutic targets.

Key Words: Lung cancer, Small cell, Genome, Comprehensive, PI3K/AKT/mTOR.

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Small cell lung cancer (SCLC) comprises approximately 15% of all lung cancers,[1] and it is an exceptionally aggressive malignancy with a high proliferative index and an unusually strong predilection for early metastasis.[2] Despite extensive basic and clinical research over the past 30 years, little progress has been made[2] in treating this disease.

A better understanding of the genomic changes in SCLC is essential to identify new therapeutic targets. Genomic analyses have revealed genetically altered therapeutic targets in lung adenocarcinoma[3–5] and squamous cell lung carcinoma.[6] However, a systematic genomic analysis of SCLC is difficult because this cancer subtype is rarely treated surgically, resulting in the lack of suitable tumor specimens for comprehensive analysis.

Two reports regarding the comprehensive genomic analysis of SCLC with a relatively small number of samples have been published recently. These reports suggested that transcriptional deregulation (i.e., via *RB1*, *SOX2*, *MYC* family members and chromatin modifiers) might play a role in SCLC biology.[7,8] However, to date, attempts to develop targeted therapies toward these transcriptional deregulations have had limited success.

Activating alterations to oncogenes, such as receptor tyrosine kinases (RTKs) and PI3K/AKT/mTOR pathway proteins,[9–13] are regarded as successful therapeutic targets. We conducted a comprehensive genomic study in over 50 SCLC cases, and we found a higher penetrance of activating alterations of the PI3K/AKT/mTOR pathway that act in a mutually exclusive manner.

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PATIENTS AND METHODS

Samples

This study was approved by the Institutional Review Board (IRB) of the National Cancer Center, Japan (IRB number: 2011-201). All data used in this study were obtained from a database at the Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Japan.

From July 1992 to March 2012, we consecutively collected 1042 SCLC cases at our hospital. Fifty-five of these cases were included in the current study based on the following criteria: a surgical resection or mediastinoscopy was performed; a re-review confirmed a pathological diagnosis of SCLC; the tumor specimens contained a minimum of 70% tumor cells; enough tissue was obtained for a comprehensive analysis; the patient did not receive any neoadjuvant treatment; and the corresponding normal tissue, which was obtained from paraffin-embedded blocks of resected lung tissue that was microscopically free of cancer cells, was also available for analysis. We analyzed the exomes of these 55 samples to assess their mutational burden.

Depending on the tissue size, three to six sections (10 μm thickness) were cut. For the tumors showing a combined SCLC and other histology, only the SCLC compartment was dissected and used for analysis. Total DNA was obtained from formalin- ($n = 43$) or methanol-fixed ($n = 12$) paraffin-embedded tumors and matched normal tissue samples. All patients (100%) were Japanese.

Among these 55 cases, four exome data sets did not meet the sequence quality requirement and were excluded from further analyses. In addition, 48 samples received copy number analysis using single nucleotide polymorphism array data.

Procedures

The detailed experimental procedures are described in the Supplemental Information section (Supplemental Digital Content 1, http://links.lww.com/JTO/A625)

Whole Exon Sequencing and Copy Number Analysis

The Absolutely RNA FFPE kit (modified protocol for DNA extraction, Agilent Technologies, Santa Clara, CA) was used to prepare the DNA. Using 1 μg of dsDNA, quantified by Quant-iT PicoGreen dsDNA Reagent and Kits (Life Technologies, Carlsbad, CA), the exome-sequencing libraries were prepared. All exomes were captured using the SureSelect Human All Exon V4+UTRs Kit (Agilent Technologies) (71 mb). The exome capture libraries were sequenced by HiSeq 2000 (Illumina, San Diego, CA) to generate 100-bp paired-end data.

The Illumina HumanOmniExpress-FFPE BeadChip assay was used to analyze the genotype, DNA copy number, and loss of heterozygosity (LOH) in 48 primary-normal paired samples. All samples, except for 1 ($n = 47$), passed our quality control metrics for sample identity and data quality. A subset of 693,000 high-quality single nucleotide polymorphisms was selected for all analyses (Supplemental Figure 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). A gene was considered copy number amplified if the calculated copy number in a sample was more than or equal to 4, and a gene was considered copy loss if the copy number in a sample was 0. Recurrent genomic regions with DNA copy gain and loss were identified using GISTIC, version 2.0.[14,15]

Identification of Significantly Mutated Genes

Significantly mutated genes were identified according to a previously reported protocol.[16] The length of the total coding sequence regions was represented as N (approximately 39.8 mb). When a patient (patient i) harbored a total of m_i single nucleotide variants (SNVs), the probability that the patient harbored SNVs in gene t (length: n) was calculated as follows:

$$P_{t,i} = 1-(1-\mathrm{m_i}/\mathrm{N})^{\mathrm{n}(t)}$$

The sum of $P_{t,i}$ in 51 samples was represented as the expected number of cases with SNVs in gene t:

$$\mathrm{P}_t = \sum_{i=1}^{51}(1-(1-\mathrm{m_i}/\mathrm{N})^{\mathrm{n}(t)})$$

The p values of the observed number were calculated using the binomial probability function with R pbinom.

Cancer Census Genes and Analysis of Hot Spot Mutations

We defined the cancer census genes as follows: 487 genes listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (release version 64; http://cancer.sanger.ac.uk/cancergenome/progects/cosmic/) and 13 genes reported by Peifer et al.[7] were considered candidate driver genes. To analyze hot spot mutations, mutation data from the SCLC cases were downloaded from the COSMIC database (release version 64 or 68).

Cell Lines and Assays

The cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) or hydrocortisone, insulin, transferrin, estradiol, and selenium (HITES) medium with 5% FBS. Then, 10,000 cells were plated in three replicates into 96-well plates. After 72 hours incubation with inhibitors, cell viability was analyzed with a WST-8 assay and a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Western blotting was performed as described in the Supplemental Information section (Supplemental Digital Content 1, http://links.lww.com/JTO/A625).

RESULTS

Patient Characteristics

The characteristics of the available patient exome data ($n = 51$) are summarized in Table 1 and Supplemental Table 3 (Supplemental Digital Content 3, http://links.lww.com/JTO/A627). Fifty patients received surgical resections and one patient received a mediastinoscopy. Forty-two patients were male and nine were female. The median age at the time of surgical resection was 67 years (range, 42–86 years). Of the 51 patients, 50 (98%) had a history of smoking, and the pathological stages were distributed as follows: stage I, 28 patients; stage II, 13

TABLE 1. Patient Characteristics

Characteristic	No. of Patients
Total	51
Gender	
Male/female	42/9
Age, years	
Median (range)	67 (42–86)
Performance status	
0/1/2	35/15/1
Smoking status	
Never/ever	1/50
Pack years	
Median (range)	47 (0–98)
Histology	
Pure SCLC/combined SCLC	40/11
Pathological stage	
I/II/III/IV	28/13/9/1
Vascular invasion	
Absent/present	8/43
Lymphatic invasion	
Absent/present/unknown	31/19/1
Pleural invasion	
Absent/present/unknown	31/19/1
Tumor diameter	
Median (range)	2.5 (1.1–13.0)

patients; stage III, nine patients; and stage IV, one patient. All patients were positive for at least one of the following neuroendocrine markers: CD56, chromogranin A, or synaptophysin.

Somatic Point Mutations

The exome capture, sequencing, and analysis of the 51 SCLC tumor–normal tissue pairs identified 10,640 protein-altering somatic mutations, including 9376 missense, 707 nonsense, and 557 protein-altering insertions and/or deletions (INDEL) (Supplemental Table 4, Supplemental Digital Content 3, http://links.lww.com/JTO/A627). The SCLC tumors had an average of 209 protein-altering SNVs (range, 41–639) per case, with a mean nonsynonymous mutation rate of 6.15 mutations per mega-base (Supplemental Figure 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). Significantly mutated genes are determined as Supplemental Table 5 (Supplemental Digital Content 3, http://links.lww.com/JTO/A627). Overall, 414 genes had a p value of less than 0.01 and 1321 genes had a p value of less than 0.05.

A description of the significantly mutated cancer census genes (p value <0.05 in our data set) is provided in Table 2. Notably, *TP53* was the most frequently mutated gene (mutation frequency of 80%, p value of 5.81E–69). The mutation frequencies and p values of cancer census genes were 39% and 6.42E–22, 16% and 0.00019, 10% and 0.0017 for *RB1*, *ROS1*, and *RET*, respectively. Mutations of histone modifiers were also recurrently identified in this study; *CREBBP* was mutated in 6% of the patients, and *EP300* was mutated in 4% of the patients (Fig. 1). Recently reported candidate driver genes were also recurrently identified in the PI3K/AKT/mTOR signaling pathway. Three patients (6%) had mutations in *PIK3CA* (one E545K, two others), and two patients (4%) had mutations in the *PTEN* C2 domain (Supplemental Figure 3, Supplemental Digital Content 2, http://links.lww.com/JTO/A626).

To validate the whole exon sequencing data, we performed Sanger sequencing for the variants including four SNVs of *PIK3CA* and *ROS1* and a deletion of *KIT* in five individual tumor samples. All the variants detected using whole exon sequencing were reproduced using conventional Sanger sequencing (Supplemental Figure 4, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). Furthermore, we designed a custom target-capturing panel containing all the coding exons of 244 genes. Four tumor samples were applied to the target resequencing, and all 41 SNVs or indels in these tumor genomes were reproducibly identified (Supplemental Table 6, Supplemental Digital Content 3, http://links.lww.com/JTO/A627).

Copy Number Analysis

Next, we applied a novel algorithm to identify the significant somatic copy number alterations (Supplemental Figure 5, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). A description of frequently amplified cancer census genes (GISTIC $-\log_{10}$ q score ≥1.50) is provided in Table 3. *MYC* family members were frequently amplified (GISTIC q scores were 2.50, 1.65, and 1.57 for *MYCL1*, *MYCN*, and *MYC*, respectively). The amplifications affected *MYCL1* (4/47 cases), *MYC* (1/47 cases), and *MYCN* (1/47 cases). All *MYC* family member amplifications (13% of cases) were mutually exclusive (Fig. 1). In addition, gene amplifications were frequently found in the PI3K/AKT/mTOR signaling pathway (GISTIC q scores were 2.45 and 1.22 for *AKT2* and *RICTOR*, respectively). The gene amplifications in PI3K/AKT/mTOR signaling were observed in *AKT2* (4/47 cases) and *RICTOR* (3/47 cases), and they were also mutually exclusive (Fig. 1). Previously reported amplifications involving *SOX2* (1/47 cases) and *KIT* (1/47 cases) were also identified.

Recurrent Mutations at the Same Position

Forty genes with recurrent somatic mutations at the same position were identified in this study and the COSMIC database (Table 4). *TP53*, the well-characterized tumor suppressor gene, had 29 different positions that mutated more than or equal to two times (total recurrent samples, 134). *RB1* had four different positions that mutated two or three times. The remaining 38 genes had one position that mutated two or three times. Well-established activating mutations in *PIK3CA*, the catalytic subunit of phosphoinositide-3 kinase (E545), were also detected in another SCLC cohort.

PI3K/AKT/mTOR Pathway Alteration

Because of the large number of somatic point mutations and focal amplifications found in the PI3K/AKT/mTOR signaling pathway (e.g., *PIK3CA*, *PTEN*, *AKT2*, and *RICTOR*), we focused our investigation on the changes in the PI3K/AKT/mTOR pathway. We observed that the PI3K/AKT/mTOR pathway was altered in 17/47 (36%) of the SCLC tumors (Fig. 1), and all altered genes in the PI3K/AKT/mTOR pathway were mutually exclusive. There was no difference in the

TABLE 2. Significantly Mutated Cancer Census Genes (*$p < 0.05$ in This Study)

	This Data Set ($n = 51$)			COSMIC Database	
Symbol	Mutated Case	Mutation Frequency (%)	p Value*	Mutated Case/Total	Mutation Frequency (%)
TP53	41	80	5.81E–69	235/308	76
RB1	20	39	6.42E–22	99/181	55
ROS1	8	16	0.00019	7/65	11
RET	5	10	0.0017	4/147	3
IKZF1	4	8	0.0017	0/57	0
CD79B	2	4	0.0042	0/61	0
PAX7	3	6	0.0074	3/63	5
HIP1	4	8	0.0076	1/62	2
CDH11	4	8	0.0086	0/62	0
MN1	4	8	0.014	1/61	2
PTEN	2	4	0.017	39/309	13
ERBB2	4	8	0.018	1/167	1
LPP	3	6	0.019	0/62	0
MLL2	6	12	0.021	7/57	12
BCL11B	3	6	0.022	0/63	0
LMO1	1	2	0.023	0/62	0
NR4A3	3	6	0.028	1/56	2
ZNF521	4	8	0.031	10/62	16
PIK3CA	3	6	0.034	38/272	14
WT1	2	4	0.040	1/127	1
TRIM33	3	6	0.041	1/62	2
PBRM1	4	8	0.042	1/61	2
FUS	2	4	0.043	0/62	0
ABL1	3	6	0.044	2/63	3
RPN1	2	4	0.045	0/57	0
BTG1	1	2	0.045	0/62	0

*p value., P binom.

clinical characteristics, such as smoking status, gender, and age, between the PI3K/AKT/mTOR pathway-affected group (Group A) and the PI3K/AKT/mTOR pathway-unaffected group (Group B). The frequencies of *TP53* and *RB1* mutations were identical between Group A and Group B. However, more *MYC* family genes tended to be amplified in Group B; Group A did not harbor *KRAS* or *BRAF* mutations, and most patients in Group A did not have MAPK/ERK pathway changes.

The correlation between the PI3K/AKT/mTOR pathway changes and RTKs is shown in Supplemental Figure 6 (Supplemental Digital Content 2, http://links.lww.com/JTO/A626). The changes in various targetable RTK genes were detected, such as *ERBB2* ($n = 4$), *KIT* ($n = 2$), *PDGFRA* ($n = 3$), *PDGFRB* ($n = 2$), *KDR* ($n = 3$), *MET* ($n = 1$), *ROS1* ($n = 8$), and *RET* ($n = 5$). However, none of these genes showed a recurrent mutation at the same point in this data set or the COSMIC database. The PI3K/AKT/mTOR pathway status did not correlate with the RTK changes.

Drug Sensitivity

To further investigate whether the PI3K/AKT/mTOR pathway could be a feasible therapeutic target in SCLC, we tested the in vitro drug sensitivity of the SCLC cell lines using the clinically developed compounds targeting this pathway (Fig. 2 and Supplemental Figure 7, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). We selected three SCLC cell lines with genetic alterations in the PI3K/AKT/mTOR pathway: H446 (*PTEN*-loss, *MYC*-amplified), H1048 (*PIK3CA* mutation), and H1694 (*AKT3*-amplified). We also examined H82 (*MYC*-amplified) and H209, which do not display activation of the PI3K/AKT/mTOR pathway. Using these cell lines, we assessed the efficacy of four compounds that inhibit the PI3K/AKT/mTOR pathway and are in on-going phase I/II trials: BEZ235 (PI3K and mTOR inhibitor), BKM120 (PI3K inhibitor), INK128 (mTOR inhibitor), and MK2206 (AKT inhibitor), as well as one cytotoxic agent, cisplatin. None of the cell lines showed apparent cytotoxicity in response to doses up to 1 μM cisplatin. Conversely, all PI3K/AKT/mTOR inhibitors significantly impaired the proliferation of the SCLC cell lines. H1048, which harbors a *PIK3CA* mutation (H1047R), was the most sensitive to all of the PI3K/AKT/mTOR inhibitors, with IC50 values of 3.8, 5.4, 99.9, and 195.4 nM for INK128, BEZ235, MK2206, and BKM120, respectively. BEZ235 was the most effective compound to specifically inhibit H1048 cell growth

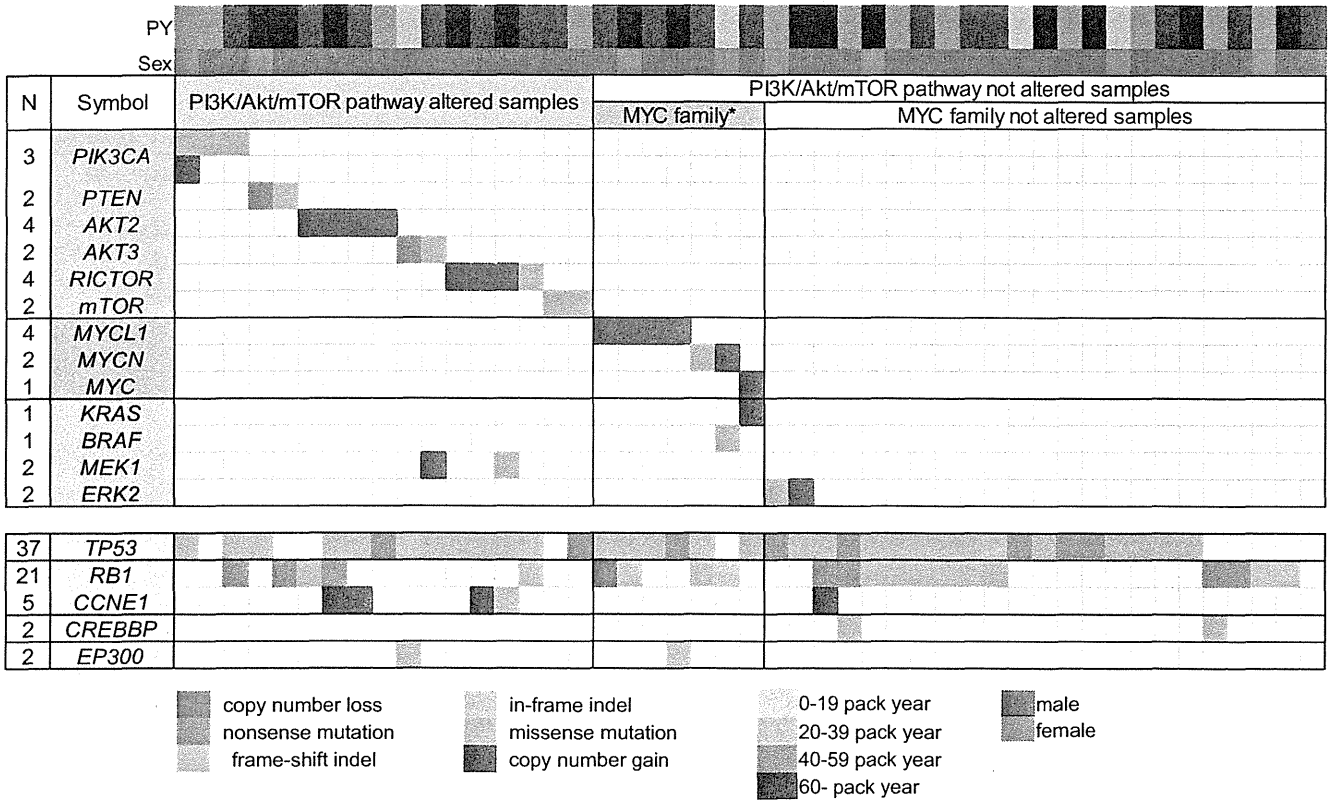

FIGURE 1. An overview of the key driver mutations and major associated clinical features of 47 SCLC samples. The number of events per gene is noted on the left. The genes are displayed as rows, and the samples are displayed as columns, with major associated clinical features.
PY, PACK YEARS; MYC FAMILY*, MYC FAMILY ALTERED SAMPLES.

TABLE 3. Frequently Mutated Cancer Census Genes ($-\log_{10}$ q score ≥1.5)

	This Data Set (n = 47)
Symbol	**$-\log_{10}$ (q score)**
MYB	3.40
MYCL1	2.50
AKT2	2.45
CLTCL1	2.34
LIFR	2.21
IL7R	2.13
THRAP3	2.09
ETV5	2.05
BCL6	1.99
EIF4A2	1.93
LPP	1.89
PAX5	1.89
ZNF384	1.86
ARID1A	1.65
MYCN	1.65
FANCG	1.65
MYC	1.57
MDS2	1.52

(IC50 = 5.4), with an IC50 value greater than 10-fold lower than that of H82 (IC50 = 58.3 nM) and fivefold lower than that of H209 (IC50 = 29.7 nM). In contrast, H446 (IC50 = 33.3 nM) and H1694 cells (IC50 = 52.5 nM) were relatively resistant to BEZ235 treatment.

The impact of BEZ235 on AKT phosphorylation in SCLC cells was investigated using Western blot analysis. AKT was activated in the H446 and H1048 cells under these culture conditions, and it was effectively inhibited after being treated with 10 nM BEZ235. Conversely, constitutive phosphorylation of AKT was not observed in H1694 cells, even when pan-AKT was over-expressed. In addition, AKT phosphorylation was not detected in the H82 and H209 cells. Regarding factors located downstream of mTOR, S6RP was phosphorylated in all five SCLC cell lines. Especially, the phosphorylation level was high in AKT-activated H446 and H1048 cells. BEZ235 significantly reduced the phosphorylation of S6RP in all the cells.

To evaluate the contribution of PI3K/AKT/mTOR signaling to SCLC cell proliferation, we used RNA interference (RNAi) to down-regulate the expression of *PIK3CA* in H1048 cells. The transient silencing of *PIK3CA* impaired the phosphorylation of AKT and S6RP (Supplemental Figure 8, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). In addition, *PIK3CA* silencing induced a decrease in the proliferation of H1048 cells.

TABLE 4. The Recurrent Mutations Detected at the Same Position in This Study and the COSMIC Database

Gene	Recurrent in This Study (no.)	No. in This Data Set	Recurrent in COSMIC Database (no.)	No. in COSMIC Database	Total
TP53	Q38 (1) T155 (1) V157 (1) R158 (2) A159 (1) M160 (1) A161 (1) Y163 (2) R175 (1) C176 (2) H179 (1) Q192 (1) D208 (1) R209 (1) R213 (1) S215 (2) Y220 (1) R248 (1) R249 (2) L265 (1) G266 (2) R273 (1) R283 (2) E286 (1) E294 (1) E298 (1) Q317 (1) R337 (2) R342 (1)	37	Q38 (1) T155 (4) V157 (11) R158 (3) A159 (1) M160 (1) A161 (1) Y163 (2) R175 (4) C176 (2) H179 (7) Q192 (2) D208 (1) R209 (2) R213 (3) S215 (1) Y220 (8) R248 (7) R249 (11) L265 (1) G266 (3) R273 (8) R283 (1) E286 (5) E294 (3) E298 (2) Q317 (1) R342 (1)	97	134
RB1	T543 (2) W78 (1) W195 (1) E322 (1)	5	W78 (1) W195 (2) E322 (1)	4	9
ABRA	S276 (2)	2		0	2
AP3M2	T72 (2)	2		0	2
CLEC4G	R23 (2)	2		0	2
DACT1	V481 (2)	2		0	2
DPP6	Q345 (2)	2		0	2
DUSP27	D886 (2)	2		0	2
GPR149	R540 (2)	2		0	2
KIAA2022	Q738 (2)	2		0	2
OR9G1	C168 (2)	2		0	2
PCDHGA5	E782 (2)	2		0	2
PDE4C	P39 (2)	2		0	2
PJA1	E182 (2)	2		0	2
ZFP1	Q287 (2)	2		0	2
B2M	M1 (1)	1	M1 (2)	2	3
PIK3CA	E545 (1)	1	E545 (2)	2	3
AFF2	D506 (1)	1	D506 (1)	1	2
ASTN1	R184 (1)	1	R184 (1)	1	2
BEND3	G263 (1)	1	G263 (1)	1	2
C8A	R438 (1)	1	R438 (1)	1	2
CREB3L3	P82 (1)	1	P82 (1)	1	2
CST4	R46 (1)	1	R46 (1)	1	2
DEFB112	R112 (1)	1	R112 (1)	1	2
GPR139	T322 (1)	1	T322 (1)	1	2
HCN1	Q772 (1)	1	Q772 (1)	1	2
ITGA4	R481 (1)	1	R481 (1)	1	2
JAM3	Y31 (1)	1	Y31 (1)	1	2
KCNJ12	R261 (1)	1	R261 (1)	1	2
LIFR	Q978 (1)	1	Q978 (1)	1	2
LRRC52	A258 (1)	1	A258 (1)	1	2
MED23	N1095 (1)	1	N1095 (1)	1	2
MUC6	P27 (1)	1	P27 (1)	1	2
OPN4	C303 (1)	1	C303 (1)	1	2
OR10S1	A283 (1)	1	A283 (1)	1	2
OR2W1	I206 (1)	1	I206 (1)	1	2
OR5F1	S265 (1)	1	S265 (1)	1	2
OR5L1	S267 (1)	1	S267 (1)	1	2
SLC28A3	Q261 (1)	1	Q261 (1)	1	2
ZNF382	C278 (1)	1	C278 (1)	1	2

no., number Recurrent (no.), positions with recurrent mutations (no. of instances).

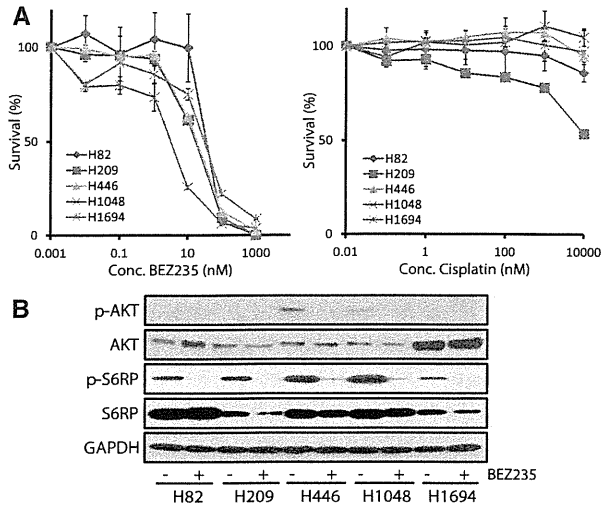

FIGURE 2. (*A*) The concentration–response cell survival curves of SCLC cell lines with or without genetic alteration in the PI3K/AKT/mTOR pathway in response to BEZ235 (nM) and Cisplatin (nM). The *PIK3CA* mutation positive cell line, H1048, is relatively sensitive to BEZ235. The H82 and H209 cell lines are negative controls. (*B*) Western blotting was used to investigate the impact of BEZ235 on AKT phosphorylation and S6RP phosphorylation in the SCLC cells. AKT was activated in H446 and H1048 cells, and it was inhibited after being treated with 10nM BEZ235. AKT was amplified but not constitutively phosphorylated in the H1694 cells. AKT phosphorylation was not detected in the negative control cell lines, H82 and H209. With regard to factors located downstream of mTOR, S6RP was phosphorylated in all five SCLC cell lines. Especially, the phosphorylation level was high in AKT-activated H446 and H1048 cells. BEZ235 significantly reduced the phosphorylation of S6RP in all the cells.

DISCUSSION

We performed an integrative genomic analysis of SCLC in Japanese patients. The SCLC tumors had a significantly high mutation rate. An analysis of the base-level transitions and transversions showed that G-to-T transversions were predominant (Supplemental Figure 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A626), which was consistent with the demonstrated effects of tobacco smoke carcinogens on DNA.[8,17] A high prevalence of inactivating mutations in *TP53* and *RB1* and recently reported candidate driver genes, including the mutations of histone modifiers (*CREBBP*[7] and *EP300*[7]), were recurrently observed along with the amplification of *MYC* family members.[7,14,18,19] These data indicate that the genomic landscape of SCLC is equivalent between Asian and Caucasian populations.[7,8,17,18]

SCLC is characterized by aggressive growth and a poor prognosis, and no single molecular targeted drug has shown any clinical efficacy over an extended period. A number of inhibitors targeting changes in RTKs are currently used in clinical use. Alterations in well-known, targetable RTK genes, such as *ERBB2*, *KIT*, *PDGFRA*, *PDGFRB*, *KDR*, *MET*, *ROS1*, and *RET*, were detected in this study. However, these alterations did not overlap with previously reported activating mutations.

The PI3K/AKT/mTOR signaling pathway is involved in the survival, proliferation, and migration of SCLC cell lines.[13] We confirmed the activation of the PI3K pathway in the SCLC-derived cell lines. AKT protein overexpression was observed in the *AKT3*-amplified H1694 cells, and phosphorylated-AKT and S6RP were increased in the *PTEN*-lacking H446 cells and *PIK3CA*-mutated H1048 cells. In addition, the significant decrease in the proliferation of H1048 cells induced by *PIK3CA* silencing suggested that the proliferation of these cells was strongly dependent on the PI3K/AKT/mTOR pathway (Supplemental Figure 8, Supplemental Digital Content 2, http://links.lww.com/JTO/A626). Consistently, genetic changes in the PI3K/AKT/mTOR pathway were detected in approximately 40% of our clinical samples. In addition to high penetrance, these alterations occurred in a mutually exclusive manner. A similar trend was observed in another Japanese cohort of primary SCLC (Supplemental Table 7, Supplemental Digital Content 3, http://links.lww.com/JTO/A627). In addition to SCLC, a significant exclusion pattern among PI3K pathway molecules was observed in the systematic analysis of breast cancer genomes.[20] Together, these data suggest indispensable roles for this pathway in tumorigenesis.

Two specific inhibitors of mTORC1, everolimus[10] and temsirolimus,[11] were tested against SCLC in a Phase II study. However, single-agent antitumor activity was limited in unselected patients; the response rate in these studies was less than 10%. To improve the response to these inhibitors, the addition of PI3K inhibition has been suggested. The dual inhibition of PI3K and mTOR might be advantageous over single inhibition by suppressing a S6K feedback loop that leads to the pathway reactivation.[21] Based on this idea, an on-going phase I study of the PI3K and mTORC1/2 dual inhibitor, BEZ235, was designed for the patients with advanced solid tumors harboring *PIK3CA* or *PTEN* alteration (NCT01195376). In this study, we showed that the survival of the cisplatin-resistant SCLC cell lines was well suppressed by BEZ235, accompanied by the suppression of S6RP phosphorylation. Notably, the effect was most significant against H1048 cells, which harbor a *PIK3CA*-activating mutation.

However, we found that not all SCLC cell lines harboring PI3K/AKT/mTOR pathway alterations exhibited a similar sensitivity to BEZ235. Although AKT phosphorylation was significantly inhibited by BEZ235 in both the H446 cells and H1048 cells, the sensitivity of the H446 cells was less than that of the H1048 cells. *MYC* gene amplification reportedly evades PI3K-targeted therapy.[22] *MYC* amplification was demonstrated in H446 cells,[23,24] and this co-alteration could be one cause of the observed low sensitivity. Thus, to determine the most beneficial concentrations for patients, both direct target molecules and other interfering signaling pathways should be simultaneously assessed. In this study, no surgically resected tumors harbored co-alterations of the PI3K/AKT/mTOR pathway and MYC gene amplification. However, the sample size of this study and other published systemic analyses remained small, and many of the samples were obtained from relatively early-stage tumors. We should expand the sample size and further analyze samples of advanced tumors using biopsy, necropsy, and autopsy specimens to clarify the coexistence of oncogenic alterations in SCLC.

Although further large-scale validation studies are needed, our data suggest that evaluating the genetic status of molecules that modify the PI3K/AKT/mTOR signaling pathway, such as MYC family and MAPK pathway molecules, is essential to select patients with potential sensitivity to PI3K/AKT/mTOR inhibitors. In other words, enriching the study population by performing the integrative genomic analysis is essential when performing phase studies of PI3K/AKT/mTOR inhibitors in SCLC.

In conclusion, the SCLC genome possesses distinguishable genetic features in the PI3K/AKT/mTOR pathway. Genetic alterations in the PI3K/AKT/mTOR pathway were noted as a top therapeutic priority in SCLC. In addition to surgically resected samples, advanced tumors should be examined for comprehensive genomic analysis.

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REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012;62:10–29.
2. William WN Jr, Glisson BS. Novel strategies for the treatment of small-cell lung carcinoma. *Nat Rev Clin Oncol* 2011;8:611–619.
3. Govindan R, Ding L, Griffith M, et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. *Cell* 2012;150:1121–1134.
4. Kohno T, Ichikawa H, Totoki Y, et al. KIF5B-RET fusions in lung adenocarcinoma. *Nat Med* 2012;18:375–377.
5. Lipson D, Capelletti M, Yelensky R, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. *Nat Med* 2012;18:382–384.
6. Cancer Genome Atlas Research Network. Comprehensive genomic characterisation of squamous cell lung cancers. *Nature* 2012; 489: 519–525.
7. Peifer M, Fernández-Cuesta L, Sos ML, et al. Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nat Genet* 2012;44:1104–1110.
8. Rudin CM, Durinck S, Stawiski EW, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet* 2012;44:1111–1116.
9. Pietanza MC, Ladanyi M. Bringing the genomic landscape of small-cell lung cancer into focus. *Nat Genet* 2012;44:1074–1075.
10. Tarhini A, Kotsakis A, Gooding W, et al. Phase II study of everolimus (RAD001) in previously treated small cell lung cancer. *Clin Cancer Res* 2010;16:5900–5907.
11. Pandya KJ, Dahlberg S, Hidalgo M, et al. A randomised, phase II trial of two dose levels of temsirolimus (CCI-779) in patients with extensive-stage small-cell lung cancer who have responding or stable disease after induction chemotherapy: a trial of the Eastern Cooperative Oncology Group (E1500). *J Thorac Oncol* 2007;2:1036–1041.
12. Voortman J, Lee JH, Killian JK, et al. Array comparative genomic hybridization-based characterization of genetic alterations in pulmonary neuroendocrine tumors. *Proc Natl Acad Sci U S A* 2010;107: 13040–13045.
13. Wojtalla A, Fischer B, Kotelevets N, et al. Targeting the phosphoinositide 3-kinase p110-α isoform impairs cell proliferation, survival, and tumor growth in small cell lung cancer. *Clin Cancer Res* 2013;19:96–105.
14. Mermel CH, Schumacher SE, Hill B, Meyerson ML, Beroukhim R, Getz G. GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol* 2011;12:R41.
15. Beroukhim R, Getz G, Nghiemphu L, et al. Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. *Proc Natl Acad Sci U S A* 2007;104:20007–20012.
16. Suzuki A, Mimaki S, Yamane Y, et al. Identification and characterization of cancer mutations in Japanese lung adenocarcinoma without sequencing of normal tissue counterparts. *PLoS One* 2013;8:e73484.
17. Pleasance ED, Stephens PJ, O'Meara S, et al. A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature* 2010;463:184–190.
18. Arriola E, Cañadas I, Arumí M, Rojo F, Rovira A, Albanell J. Genetic changes in small cell lung carcinoma. *Clin Transl Oncol* 2008;10:189–197.
19. Iwakawa R, Takenaka M, Kohno T, et al. Genome-wide identification of genes with amplification and/or fusion in small cell lung cancer. *Genes Chromosomes Cancer* 2013;52:802–816.
20. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012; 490: 61–70.
21. Gadgeel SM, Wozniak A. Preclinical rationale for PI3K/Akt/mTOR pathway inhibitors as therapy for epidermal growth factor receptor inhibitor-resistant non-small-cell lung cancer. *Clin Lung Cancer* 2013;14:322–332.
22. Ilic N, Utermark T, Widlund HR, Roberts TM. PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis. *Proc Natl Acad Sci U S A* 2011;108:E699–E708.
23. Brennan J, O'Connor T, Makuch RW, et al. myc family DNA amplification in 107 tumors and tumor cell lines from patients with small cell lung cancer treated with different combination chemotherapy regimens. *Cancer Res* 1991;51:1708–1712.
24. Sos ML, Dietlein F, Peifer M, et al. A framework for identification of actionable cancer genome dependencies in small cell lung cancer. *Proc Natl Acad Sci U S A* 2012;109:17034–17039.

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Clinical Cancer Research

Personalized Medicine and Imaging

A Three-microRNA Signature Predicts Responses to Platinum-Based Doublet Chemotherapy in Patients with Lung Adenocarcinoma

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Abstract

Purpose: To examine the clinical utility of intratumor microRNAs (miRNA) as a biomarker for predicting responses to platinum-based doublet chemotherapy in patients with recurring lung adenocarcinoma (LADC).

Experimental Design: The expression of miRNAs was examined in LADC tissues surgically resected from patients treated with platinum-based doublet chemotherapy at the time of LADC recurrence. Microarray-based screening of 904 miRNAs followed by quantitative reverse transcription-PCR–based verification in 40 test cohort samples, including 16 (40.0%) responders, was performed to identify miRNAs that are differentially expressed in chemotherapy responders and nonresponders. Differential expression was confirmed in a validation cohort ($n = 63$ samples), including 18 (28.6%) responders. An miRNA signature that predicted responses to platinum-based doublet chemotherapy was identified and its accuracy was examined by principal component and support vector machine analyses. Genotype data for the *TP53*-Arg72Pro polymorphism, which is associated with responses to platinum-based doublet chemotherapy, were subsequently incorporated into the prediction analysis.

Results: A signature comprising three miRNAs (miR1290, miR196b, and miR135a*) enabled the prediction of a chemotherapeutic response (rather than progression-free and overall survival) with high accuracy in both the test and validation cohorts (82.5% and 77.8%). Examination of the latter was performed using miRNAs extracted from archived formalin-fixed paraffin-embedded tissues. Combining this miRNA signature with the *TP53*-Arg72Pro polymorphism genotype marginally improved the predictive power.

Conclusion: The three-miRNA signature in surgically resected primary LADC tissues may by clinically useful for predicting responsiveness to platinum-based doublet chemotherapy in patients with LADC recurrence. *Clin Cancer Res; 20(18); 4784–93. ©2014 AACR.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Introduction

Lung adenocarcinoma (LADC) is the most common type of non–small cell lung cancer (NSCLC) and is a leading cause of cancer mortality worldwide (1). Surgical resection is the best curative treatment for NSCLC; however, patients that experience recurrence after surgery and those with advanced disease receive chemotherapy to slow tumor growth and improve survival. LADCs harboring an *EGFR* mutation or an *ALK* fusion are primarily treated with specific tyrosine kinase inhibitors (TKI), with response rates of approximately 60% (2, 3). Other oncogene aberrations, such as *BRAF*, *HER2*, *RET*, and *ROS1*, are also targeted by specific TKIs (4–6), but a major barrier to curative treatment of LADC using TKIs is innate and acquired drug resistance (7, 8). Furthermore, more than 60% of USA/European and more than 30% of Japanese LADC cases do not harbor the oncogene aberrations listed above (9, 10). Such resistant and oncogene-negative LADC cases are treated with

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Translational Relevance

The use of biomarkers to identify patients that will respond to platinum-based doublet chemotherapy before treatment is a critical strategy for improving the efficacy of chemotherapy for lung adenocarcinoma (LADC). Here, we report that the expression profile of three miRNAs in surgically resected primary tissues is clinically useful for predicting responsiveness to platinum-based doublet chemotherapy in patients with LADC recurring after initial surgical resection. This three-miRNA signature may be useful for the clinical management of LADC.

chemotherapy. The standard regimens comprise platinum-based doublets, that is, a combination of platinum and another agent; the drugs paired with platinum (cisplatin or carboplatin) include microtubule-targeted agents (paclitaxel, docetaxel, or vinorelbine) and DNA-damaging agents (gemcitabine or irinotecan). A series of trials in unselected patients revealed that the efficacy of each combination is similar, with response rates of 30% to 40% (11–13); thus, identifying biomarkers that can discriminate between patients that will respond to platinum-based doublet chemotherapy and those who may not before treatment will help improving the efficacy of chemotherapy for LADC.

microRNAs (miRNA) are small noncoding RNAs that posttranscriptionally regulate the translation of target genes; these miRNAs show altered expression in a variety of cancers and can modify the malignant properties of cancer cells, including the response to DNA damage (14–16). In fact, functional studies in LADC cell lines have identified a number of miRNAs that modulate sensitivity to platinum-based agents (17–23). Furthermore, miRNAs are stable in formalin-fixed paraffin-embedded (FFPE) tissues, that is, materials used for daily pathologic diagnosis (24, 25). Indeed, intratumor miRNA expression is a promising prognostic marker in patients that have undergone surgical resection (26–31), but few studies have examined the utility of miRNAs as a predictor of chemotherapeutic responses (32). To the best of our knowledge, only two such studies have been reported: one shows that a two-miRNA signature (miR149 and miR375) is associated with responses to platinum-based chemotherapy in NSCLC (n = 38), and the other shows that miR92a-2* expression is associated with chemoresistance in small-cell lung cancer (n = 34; refs. 33, 34).

Here, we investigated the utility of intratumor miRNAs as a biomarker for predicting responses to platinum-based doublet chemotherapy. We examined the expression of miRNAs in 103 surgically resected specimens obtained from patients who received platinum-based chemotherapy upon LADC recurrence to ascertain whether miRNA expression in primary tumors could predict responses to platinum-based doublet chemotherapy in patients who experienced LADC recurrence after surgery. First, a two-step screening process involving 904 miRNAs was performed to identify an miRNA signature with predictive value. The first step was performed using a test cohort comprising 40 frozen tumor samples from which RNAs were isolated, and the second step was performed using a validation cohort comprising 63 cases, for which RNAs from FFPE tissues were available. We identified a three-miRNA signature that predicted responses to platinum-based doublet chemotherapy with an accuracy of >75%.

Materials and Methods

Materials

Of note, 103 surgically resected LADC tissues were examined in the present study (Fig. 1A). Briefly, 643 Japanese patients with NSCLC received platinum-based doublet chemotherapy at the National Cancer Center Hospital (NCCH; Tokyo, Japan), between 2000 and 2008, and the therapeutic response was evaluated using the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines (35). None of the patients had received prior treatment with platinum-based chemotherapy. Of the 643 cases, 118 were recurrent cases that had undergone surgical resection at NCCH, and all were pathologically diagnosed with adenocarcinoma. Tumor tissues for RNA extraction were available for 103 of 118 cases; these cases were examined in the present study. Information regarding age, gender, pathologic TNM stage (the 7th classification), smoking habits, postoperative chemotherapy regimens and responses to platinum doublet

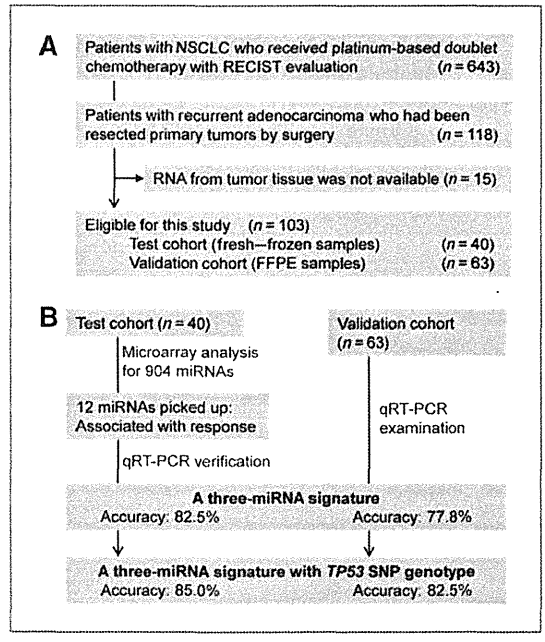

Figure 1. Patients and treatment strategy. A, selection of eligible cases, that is, 103 surgically resected cases that received platinum-based doublet chemotherapy upon LADC recurrence. B, identification and evaluation of a three-miRNA signature for the prediction of responses to chemotherapy.

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therapy, and performance status (PS) were retrospectively collected. RNAs isolated from fresh-frozen tissues were available for 40 of 103 cases; these were defined as the test cohort. The RNAs from the test cohort were subjected to miRNA microarray analysis followed by verification by quantitative reverse transcriptase-PCR (qRT-PCR) analysis. RNAs from FFPE tissues were available for the remaining 63 cases; these were defined as the validation cohort. In addition, patients were classified into two categories according to RECIST guidelines: those that responded to platinum-based doublet chemotherapy [complete response (CR) or partial response (PR)] and those that did not [stable disease (SD) or progressive disease (PD)].

RNA extraction

RNA was extracted from snap-frozen tissues (test cohort) using TRizol reagent (Thermo Fisher Scientific). The quality and quantity of the RNAs were examined using a Bioanalyzer (Agilent). All RNA samples showed an RNA integrity number RIN >6.0; therefore, they were subjected to microarray analysis. For the validation cohort, RNA was isolated from three unstained FFPE sections (5-μm thick). The area of the carcinoma in the three unstained sections was outlined by referring to a sequential section that was stained with hematoxylin and eosin. Each marked area was macrodissected using a sterile disposable scalpel and RNA was isolated using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion). Total RNA was quantified using a NanoDrop ND-1000 spectrometer (Thermo Fisher Scientific). The optical density (OD) 260/280 and OD 260/230 ratios were used for quality control.

Microarray experiments

The Human miRNA Microarray Kit release 14 (8 × 15 K; Agilent Technologies), covering 904 miRNAs, was used to screen for miRNAs in the test cohort samples ($n = 40$). Data were normalized and analyzed using GeneSpring GX software (version 12.5; TOMY Digital Biology). The fold change in expression was defined as the ratio of expression in responders to that in nonresponders. Normalized and raw expression data were deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information (GSE56264).

Examination of driver oncogene aberrations

All 40 test cohort samples were also screened for oncogene fusions (*EML4*– and *KIF5B–ALK*, *KIF5B*– and *CCDC6–RET*; and *CD74*–, *EZR*–, and *SLC34A2–ROS1*) by reverse transcription-PCR as previously described (4, 36). Genomic DNA was extracted from fresh or frozen samples from all 103 subjects using a QIAamp DNA Mini Kit (QIAGEN) and then analyzed for *EGFR*, *KRAS*, *BRAF*, and *HER2* hot spot mutations using the high resolution melting method (37, 38).

qRT-PCR analysis

qRT-PCR of mature miRNA was performed using TaqMan MicroRNA assays (Thermo Fisher Scientific) and the 7900 HT Fast Real-Time PCR System (Thermo Fisher Scientific). cDNA was synthesized using miRNA-specific primers and a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). RNA (40 ng) was reverse transcribed in a 20 μL reaction containing gene-specific RT probes. All assays were performed in triplicate and investigators were blinded to the clinical outcome. All TaqMan probes were purchased from Thermo Fisher Scientific: hsa-miR135a-3p (ID 002232), hsa-miR196b-5p (ID 002215), hsa-miR1181 (Assay ID 241045_mat), hsa-miR31-5p (ID 002279), hsa-miR31-3p (ID 002113), hsa-miR1290 (ID 002863), hsa-miR598 (ID 001988), hsa-miR1 (ID 002222), hsa-miR144-5p (ID 002148), hsa-miR628-5p (ID 002433), hsa-miR449a (ID 001030), and hsa-miR34b-3p (ID 002102). RNU66 (ID 001002) was used as a normalization control. Relative expression of miRNAs was calculated using RQ manager 1.2 (Thermo Fisher Scientific).

Statistical analysis

Differences in miRNA expression levels between responders and nonresponders were tested by the Mann–Whitney *U* test using Graphpad Prism v5.0 (Graphpad Software Inc). Spearman correlation analysis was used to examine the correlation between microarray and qRT-PCR data (Graphpad Prism v5.0). Linear discriminant analysis was performed for each cohort to distinguish responders from nonresponders (JMP 10 software; SAS Institute) based on miRNA expression (i.e., the miRNA signature). Continuous expression values for a single miRNA or for plural miRNAs, that is, ΔC_t values obtained by qRT-PCR against RNU66, were included as variables in the analysis. Receiver operating characteristic (ROC) curves were generated to evaluate response sensitivity and the area under the curve (AUC) was calculated (JMP 10). Principal component analysis (PCA) of the expression of three miRNAs (miR1290, miR196b, and miR135a*) was performed using JMP 10.

Results

Sample selection

The aim of this study was to identify biomarkers in patients with metastatic LADC who relapsed following potential curative surgical resection. Therefore, surgically resected primary tumor tissues from 103 LADC patients who were treated with platinum-based doublet chemotherapy upon recurrence were selected for miRNA profiling (Fig. 1A). The cases were assigned to a test cohort ($n = 40$; RNAs from frozen tissue available) or a validation cohort ($n = 63$; RNAs from FFPE tissues available) according to the availability of tumor tissue samples. Patients in both cohorts were classified as responders (CR and PR) or nonresponders (SD and PD) to platinum-based doublet chemotherapy according to the RECIST criteria (Materials and Methods; Supplementary Table S1). In this study, platinum-based doublet chemotherapy includes several different regimens. The cohorts were similar in terms of clinicopathologic characteristics such as age, gender, smoking habits, pathologic stage, representative oncogene mutations, therapeutic regimen, and therapeutic response (Table 1). The samples

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Table 1. Clinicopathologic characteristics on surgically resected primary lung ADC

	Test cohort ($n = 40$)	Validation cohort ($n = 63$)	*P*
Age, y			0.20
Mean (range)	59 (47–67)	59 (32–72)	
Gender number (%)			
Male	23 (58)	28 (44)	0.23
Female	17 (42)	35 (56)	
Smoking (pack year) number (%)			
Never smoker	16 (40)	33 (52)	0.09
<20	3 (8)	10 (16)	
≥20	21 (52)	20 (32)	
TP53 genotype number (%)			0.49
Arg/Arg	20 (50)	24 (38)	
Arg/Pro	16 (40)	33 (52)	
Pro/Pro	4 (10)	6 (10)	
Driver oncogene mutation number (%)			
Negative	14 (35)	28 (44)	0.34
Positive	26 (65)	35 (56)	
EGFR mutation	19	28	
KRAS mutation	3	7	
HER2 mutation	3	0	
BRAF mutation	1	0	
Driver oncogene fusion number (%)			
Negative	36 (90)	NE	
Positive	4 (10)	NE	
ALK fusion	2	NE	
RET fusion	0	NE	
ROS1 fusion	2	NE	
TNM stage at initial diagnosis number (%)			0.83
IA	6 (15)	5 (8)	
IB	5 (13)	12 (19)	
IIA	4 (10)	10 (16)	
IIB	2 (5)	2 (3)	
IIIA	18 (45)	26 (41)	
IIIB	2 (5)	0	
IV	3 (8)	8 (13)	
TNM stage at recurrence number (%)			0.32
IA	0	0	
IB	0	0	
IIA	0	0	
IIB	0	0	
IIIA	0	0	
IIIB	2 (5)	3 (5)	
IV	38 (95)	60 (95)	
Recurrent portion			1
Local/regional	2 (5)	3 (5)	
Metastasis	38 (95)	60 (95)	
M1a	22	28	
M1b	16	32	
Platinum-based regimens number (%)			0.44
Platinum + paclitaxel	30 (75)	42 (67)	
Platinum + gemcitabine	9 (23)	14 (22)	

(Continued on the following page)