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

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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

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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

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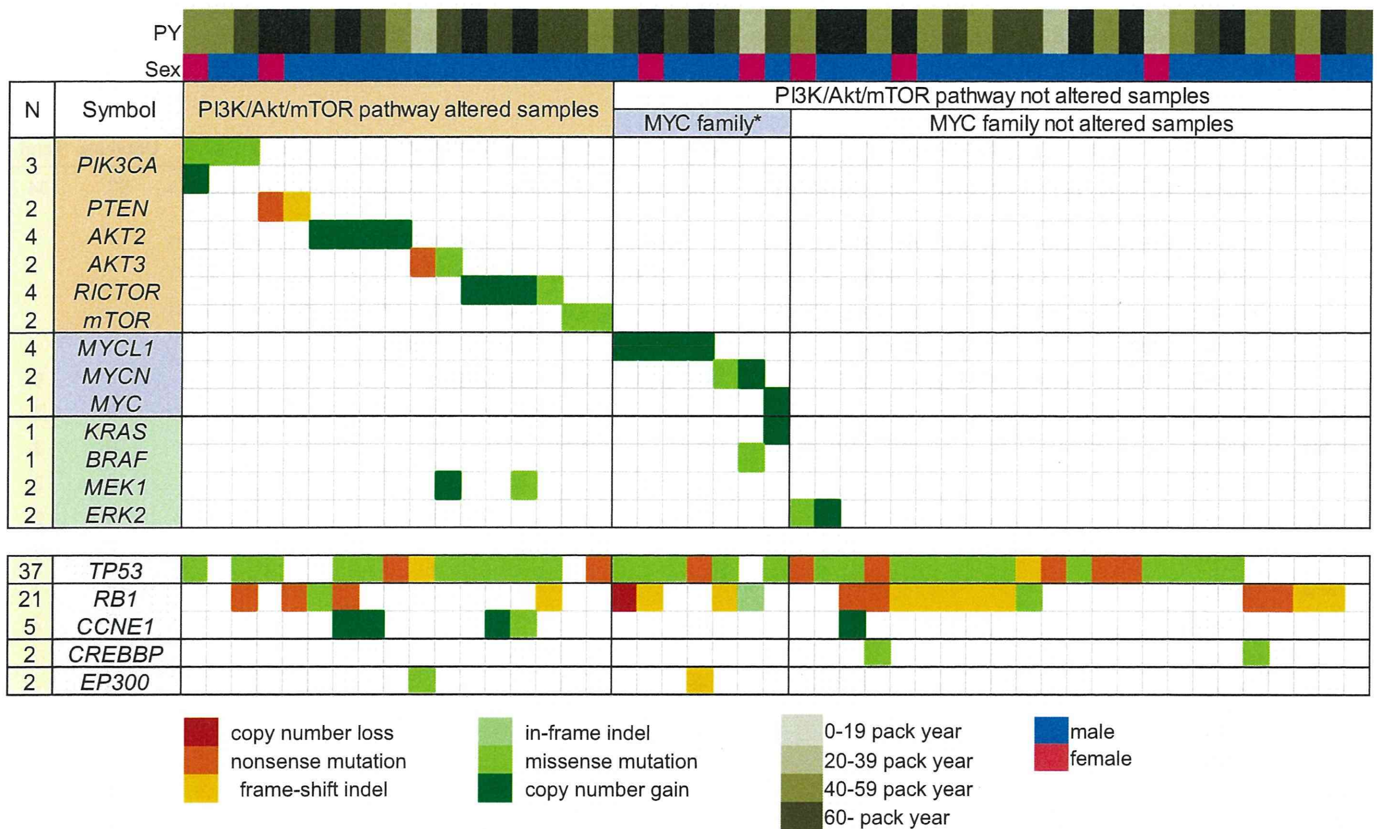

FIGURE 1. An overview of the key driver mutations and major associated clinical features of 47 SCLC samples. The number of events per gene is noted on the left. The genes are displayed as rows, and the samples are displayed as columns, with major associated clinical features.
PY, PACK YEARS; MYC FAMILY*,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

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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)	37	Q38 (1) T155 (4) V157 (11) R158 (3) A159 (1) M160 (1)	97	134
	A161 (1) Y163 (2) R175 (1) C176 (2) H179 (1) Q192 (1)		A161 (1) Y163 (2) R175 (4) C176 (2) H179 (7) Q192 (2)		
	D208 (1) R209 (1) R213 (1) S215 (2) Y220 (1) R248 (1)		D208 (1) R209 (2) R213 (3) S215 (1) Y220 (8) R248 (7)		
	R249 (2) L265 (1) G266 (2) R273 (1) R283 (2) E286 (1)		R249 (11) L265 (1) G266 (3) R273 (8) R283 (1) E286 (5)		
	E294 (1) E298 (1) Q317 (1) R337 (2) R342 (1)		E294 (3) E298 (2) Q317 (1) R342 (1)		
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).

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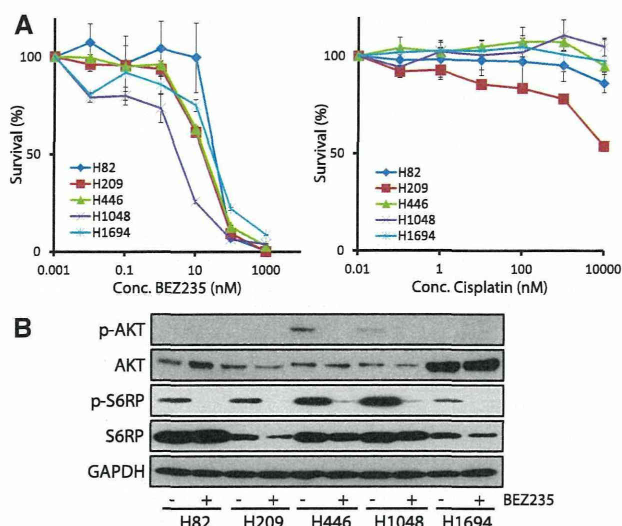

FIGURE 2. (*A*) The concentration–response cell survival curves of SCLC cell lines with or without genetic alteration in the PI3K/AKT/mTOR pathway in response to BEZ235 (nM) and Cisplatin (nM). The *PIK3CA* mutation positive cell line, H1048, is relatively sensitive to BEZ235. The H82 and H209 cell lines 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.

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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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Low-dose Irinotecan as a Second-line Chemotherapy for Recurrent Small Cell Lung Cancer

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Objective: Irinotecan is a potent inhibitor of deoxyribonucleic acid topoisomerase 1 and the weekly schedule of 100–125 or 350 mg/m^2 administration on Day 1 every 3 weeks is recommended for recurrent small cell lung cancer. However, severe gastrointestinal toxic effects and myelosuppression are often observed in this dose setting. We conducted a retrospective study to evaluate the efficacy and safety of low-dose irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) as second-line chemotherapy for small cell lung cancer.
Methods: The medical charts of small cell lung cancer patients who had received second-line chemotherapy at the National Cancer Center Hospital East between April 2003 and June 2012 were reviewed. Consecutive 57 patients who were treated with low dose of irinotecan (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) were analyzed in this study.
Results: Median age was 70 years (range, 51–83). Fifty-two (91%) were male, 36 (63%) had an Eastern Cooperative Oncology Group performance status 0–1 and 26 (46%) were sensitive relapse. The median number of chemotherapy cycles was 2. The objective response rate was 32% (95% confidence interval: 20–45%).The median progression-free survival and the median overall survival were 2.9 months (95% confidence interval: 1.9–3.4 months) and 5.3 months (95% confidence interval: 3.6–7.6 months), respectively. The incidence of Grade 3/4 neutropenia, diarrhea and nausea/vomiting was 21, 4 and 5%, respectively.
Conclusions: Low-dose irinotecan monotherapy for recurrent small cell lung cancer might be effective with favorable toxicity. Randomized trial of 60 mg/m^2 versus standard dose of irinotecan is warranted.

Key words: small cell lung cancer – second-line – irinotecan – sensitive relapse – refractory relapse

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INTRODUCTION

Lung cancer remains the leading cause of cancer-related deaths worldwide (1). Small cell lung cancer (SCLC) accounts for ~10–15% of all types of lung cancer. Despite a high sensitivity to the initial therapy, the majority of patients develop disease recurrence. The treatment options for patients with recurrent SCLC remain limited (2–4).

Irinotecan is a potent inhibitor of DNA topoisomerase 1 (5) and has been reported to be active in recurrent SCLC patients in several Phase II trials. Patients were treated with irinotecan at a dosage of 100–125 mg/m^2 weekly or 350 mg/m^2 on Day 1 every 3 weeks in these studies (6–9). However, severe gastrointestinal toxicities and myelosuppression were often observed at these dose settings.

Actually, the dose setting of 100 mg/m^2 of irinotecan monotherapy weekly is based on Phase I trials for non-SCLC patients (10), and this dose setting was also adopted in Phase II trials for recurrent SCLC patients in Japan (6). Because

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SCLC is one of the most chemosensitive solid tumors, lower-dose irinotecan regimens might have the benefit of being less toxic, compared with the recommended dose in previous reports. In terms of dosage of irinotecan with weekly schedule, another Phase I study in France was reported (11). In the study, no Grade 2 or more serious diarrhea was observed at the dosage of 50 and 66 mg/m^2. On the other hand, prolonged diarrhea (defined as >4 days in the study) was observed two (one with Grade 2 and the other with Grade 3) of four patients at the dosage of 75 mg/m^2. In addition, irinotecan combination therapy is effective in a first-line setting and the recommended dose of irinotecan when used in combination with a platinum agent was 60 mg/m^2 on Days 1, 8 and 15 every 4 weeks (12).

Based on these findings, we have adopted a low-dose irinotecan monotherapy regimen (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) as a treatment option for recurrent SCLC in our clinical practice at the National Cancer Center Hospital East. In this retrospective study, we evaluated the efficacy and safety of irinotecan monotherapy at this dosage.

PATIENTS AND METHODS

Patient Selection

This retrospective cohort study was approved by the ethical review committee of the National Cancer Center, Tokyo, Japan. We retrospectively reviewed the medical records of patients with SCLC who were treated at the National Cancer Center Hospital East between April 2003 and June 2012. Patients were selected for this study according to the following criteria: (i) patients with a histological or cytological diagnosis of SCLC, (ii) patients with refractory or relapsed SCLC after initial chemotherapy or chemoradiotherapy and (iii) patients who were treated with a low dose of irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) as a second-line chemotherapy. For sensitive relapse SCLC, topotecan monotherapy is the standard care for sensitive relapse SCLC in second-line setting (2,3) and intravenous topotecan for five consecutive days at a dose of 1.0 mg/m^2 is approved in Japan (13). However, high frequency of serious myelotoxicity was reported in the previous studies with this treatment (3,13,14). In our clinical practice, we determined the optimal second-line regimen in each patient taking its toxicity profile and patient's feasibility into consideration. Although the application criteria of low-dose irinotecan regimen were not defined strictly due to the retrospective study from clinical practice, we generally applied the low-dose irinotecan monotherapy when we were concerned about the myelotoxicities in each patient.

Data Collection

We collected clinical data from the medical records, including the patient age, sex, initial chemotherapy regimen, sensitivity to initial therapy, Eastern Cooperative Oncology Group performance status (PS) at recurrence, clinical stage (limited disease or extensive disease according to the International Association for the Study of Lung Cancer's consensus report) at disease progression and type of relapse (sensitive relapse defined as relapse at an interval of 90 days after the completion of initial chemotherapy and refractory relapse defined as no response to initial chemotherapy or relapse within 90 days after the completion of initial chemotherapy).

Treatment

Irinotecan was administered on Days 1, 8 and 15 via a 90-min intravenous infusion at a dose of 60 mg/m^2. The treatment cycles were repeated every 4 weeks. When the leukocyte or platelet count was inappropriate or diarrhea occurred on Day 8 or 15, the treatment was skipped.

Evaluation of Response and Toxicity

The objective tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors guideline, version 1.1 (15). Efficacy evaluation schedules were not defined strictly due to the retrospective study from clinical practice. However, we performed chest computed tomography (CT) evaluation every 2–3 months in principle with our clinical practice. The investigator (M.M.) reviewed all radiological imaging and re-evaluated tumor responses in each patient. In the study, confirmation of complete response (CR) and partial response (PR) were not performed. Toxicity was assessed according to the Common Terminology Criteria for Adverse Events (v4.0). The data of toxicity were collected minutely from medical records, and we could obtain each toxicity profile weekly due to the weekly schedule regimen.

Statistical Analysis

The overall survival (OS) and progression-free survival (PFS) times were estimated using the Kaplan–Meier method. The PFS was measured from the date of the start of irinotecan chemotherapy to the documented date of disease progression, death or the last follow-up. The OS was measured from the date of the start of irinotecan chemotherapy to the date of death or the last follow-up. The statistical analyses were performed using JMP software, version 10.0.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient Characteristics

Between April 2003 and June 2012, a total of 321 patients with recurrent SCLC received second-line chemotherapy. Among them, 57 consecutive patients with recurrent SCLC were treated with a low dose of irinotecan as a second-line therapy. The patient characteristics are listed in Table 1. The median age was 70 years (range, 51–83 years). The majority

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Table 1. Patient characteristics ($n = 57$)

	Number of patients (%)
Age, median (range)	70 (51–83)
Sex	
Male	52 (91)
Female	5 (9)
ECOG PS	
0	5 (9)
1	31 (54)
2	17 (30)
3	4 (7)
Type of relapse	
Refractory relapse	31 (54)
Sensitive relapse	26 (46)
Stage at recurrence	
Limited disease	13 (23)
Extensive disease	44 (77)
First-line chemotherapy	
ETP + CDDP	27 (47)
ETP + CBDCA	26 (46)
CPT + CDDP	2 (3)
CPT + ETP + CDDP	1 (2)
AMR + CDDP	1 (2)
Thoracic irradiation	
−	33 (58)
+	24 (42)
PCI	
−	44 (77)
+	13 (23)

ECOG PS, Eastern Cooperative Oncology Group performance Status; ETP, etoposide; CDDP, cisplatin; CBDCA, carboplatin; CPT, irinotecan; AMR, amrubicin; PCI, prophylactic cranial irradiation.

of patients were male (91%). In total, 158 cycles were administered. The median number of treatment cycles was 2 (range, 1–8). Of the total 158 cycles, 85 cycles (54%) were performed without skipping an administration. Sixty-four cycles (41%) were performed with the skipping of one administration on Day 8 or 15. Only nine cycles (5%) were performed with the skipping of both administrations on Days 8 and 15. The major reasons for the skipping of administration on Day 8 or 15 were leukopenia, diarrhea and fatigue.

Efficacy

The objective response rate (ORR) is shown in Table 2. Eighteen PRs were obtained, and the ORR was 32% (95% confidence interval (CI): 20–45%). The disease control rate was 58% (95% CI, 44–71%). We performed a subgroup analysis of the tumor response according to the type of relapse (sensitive relapse versus refractory relapse). The ORRs in patients with sensitive relapse and refractory relapse were 38% (95% CI, 20–59%) and 26% (95% CI, 12–45%), respectively. At the time of analysis, 56 patients (98%) had experienced disease progression and 47 (82%) patients had died. The median PFS and the median OS were 2.9 months (95% CI, 1.9–3.4 months) and 5.3 months (95% CI: 3.6–7.6 months), respectively (Fig. 1). The median PFS in patients with sensitive relapse and refractory relapse was 3.7 months (95% CI: 2.8–4.2 months) and 2.2 months (95% CI, 1.5–3.1 months), respectively. The median OS in patients with sensitive relapse and refractory relapse was 5.9 months (95% CI,

Table 2. Tumor response

	n (%)
CR	0 (0)
PR	18 (32)
SD	15 (26)
PD	19 (33)
NE	5 (9)
Response rate (95% CI)	32% (20–45%)
Disease control rate (95% CI)	58% (44–71%)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

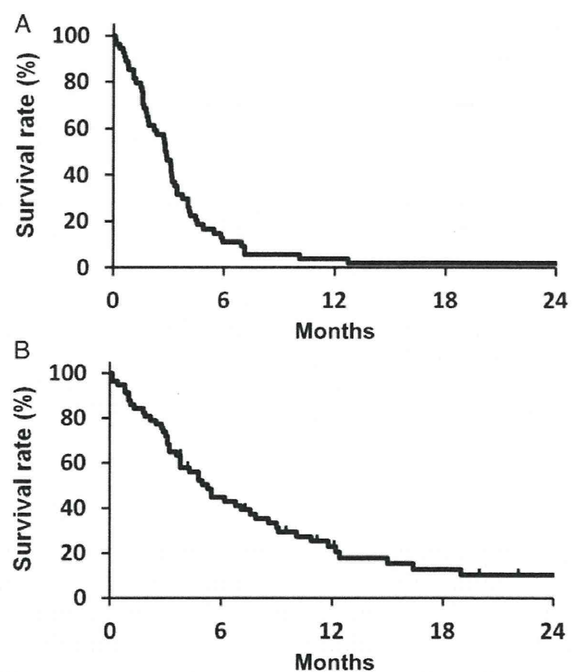

Figure 1. (A) Progression-free survival (PFS) and (B) overall survival (OS) ($n = 57$).

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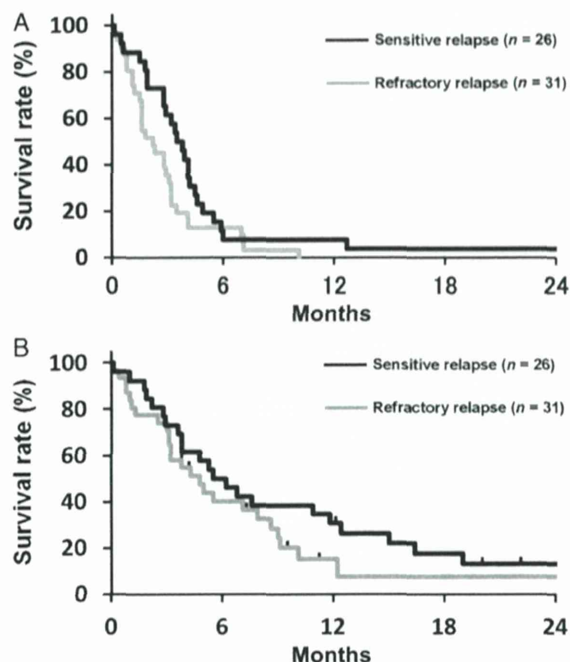

Figure 2. (A) PFS in patients with sensitive relapse and refractory relapse. (B) OS in patients with sensitive relapse and refractory relapse.

Table 3. Safety

	Grade				Grades 3–4
	1	2	3	4	% of patients
Leukopenia	20	15	10	1	19
Neutropenia	8	15	8	4	21
Thrombocytopenia	7	2	3	0	5
Diarrhea	14	14	1	1	4
Fatigue	12	4	2	–	4
Nausea/vomiting	28	4	3	0	5
Anorexia	12	11	4	0	7
Febrile neutropenia	–	–	1	1[a](Gr5)	4 (Gr3–5)
ILD	0	0	0	1[b](Gr5)	2 (Gr3–5)
Edema	0	1	0	0	0
Fever	1	0	0	0	0
Neuropathy	0	0	0	0	0
Rash	0	0	0	0	0
AST increased	2	0	0	0	0
ALT increased	2	0	0	0	0
Cre increased	0	1	0	0	0

Gr, grade; ILD, interstitial lung disease; ALT, alanine transaminase; AST, aspartate transaminase; Cre, creatinine.
[a]Grade 5 febrile neutropenia was observed in one patient.
[b]Grade 5 ILD was observed in one patient.

3.6–11.8 months) and 4.8 months (95% CI, 3.1–7.9 months), respectively (Fig. 2). In addition, the median OS according to PS was 7.1 months (95% CI: 3.8–10.1 months) with PS0-1 and 3.2 months (95% CI, 1.8–4.8 months) with PS2-3.

Safety

The worst grades of adverse events are listed in Table 3. The most common adverse events were neutropenia (all grades, 61%), nausea/vomiting (all grades, 61%) and diarrhea (all grades, 53%). The incidence of Grade 3/4 neutropenia, diarrhea and nausea/vomiting was 21, 4 and 5%, respectively. Treatment-related death was observed in two patients. One of them developed febrile neutropenia, resulting in septic shock. The other patient died of respiratory failure despite receiving methylprednisolone pulse treatment. We judged this adverse event as Grade 5 interstitial lung disease possibly related to irinotecan because congestive heart failure and bacterial pneumonia were clinically excluded by a chest X-ray and chest CT scan findings, in addition to the absence of symptoms such as systematic edema, fever and/or yellow sputum.

DISCUSSION

In this study, low-dose irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) achieved an ORR of 32% and a median PFS of 2.9 months, with a favorable toxicity profile. The incidence of Grade 3/4 neutropenia was 21%, and the incidences of Grade 3/4 diarrhea and nausea/vomiting were 4 and 5%, respectively. Several Phase II trials showed that irinotecan monotherapy (100–125 mg/m^2 weekly or 350 mg/m^2 on Day 1 every 3 weeks) was promising in a second-line setting for recurrent SCLC (6–9). An ORR of 16–47% was achieved in these studies, but these dose settings of irinotecan are associated with a high incidence of Grade 3/4 neutropenia (25–58%), diarrhea (7–37%) and nausea/vomiting (13–22%). The review of efficacy and safety data of previous studies and our study is summarized in Table 4. Ando et al. (16) reported that polymorphisms of UDP-glucuronosyltransferase (UGT) 1A1 were associated with severe neutropenia and/or diarrhea caused by irinotecan. The significance of UGT1A1 polymorphisms for predicting severe neutropenia was confirmed in an irinotecan dose setting of weekly 100 mg/m^2, biweekly 150 mg/m^2 and 350 mg/m^2 on Day 1 every 3 weeks (17,18). On the other hand, no difference in the toxicity profile was observed according to UGT1A1 polymorphisms in a dose setting of weekly 70 mg/m^2 irinotecan combined with 25 mg/m^2 of docetaxel (19). In the present study, we could not evaluate the UGT1A1 polymorphism status because this study analyzed a retrospective cohort. However, our low-dose irinotecan setting (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) might reduce the risk of severe toxicity caused by genetic polymorphisms of UGT1A1.

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Table 4. Efficacy and safety data of previous studies and our study

Authors	*n*	% of SR Pts	Dose (mg/m^2)	Schedule	RR (%)	Grade 3/4 (%)		
						Neutropenia	Diarrhea	Nausea/vomiting
Masuda et al. (6)	15	93	100	Weekly	47	33[a]	7	13
Negoro et al. (7)[b]	27	–	100	Weekly	33	25[a]	18	18
DeVore et al. (8)	44	39	125	d1.8.15.22 q6weeks	16	27	27	–/9[c]
Le Chevalier (9)	32	–	350	d1, q3weeks	16	58	37	22
Current study	57	46	60	d1. 8. 15, q4weeks	32	21	4	5

SR, sensitive relapse; Pts, patients; RR, response rate.
[a]The rate of leukopenia.
[b]Evaluation for incidence of toxicity was performed in a total of 146 lung cancer patients, including 27 previously treated small cell lung cancer patients.
[c]The rate of vomiting.

A higher dose does not always result in a higher efficacy for highly chemosensitive malignant tumors. In patients with recurrent SCLC patients, amrubicin, a topoisomerase II inhibitor, exhibited favorable activity with lower dose than standard dose decided by initial Phase I studies (20). In addition, in patients with ovarian cancer that is highly chemosensitive as well as SCLC, lower-dose topotecan (topoisomerase I inhibitor as well as irinotecan) might have equal activity compared with the standard dose of topotecan (21,22). Our results suggest that a lower dose of irinotecan might have an equal efficacy with a less toxic profile for patients with recurrent SCLC.

The median OS of 5.3 months in our study seems shorter compared with recently reported Japanese studies for recurrent SCLC. In these studies, the median OS were ranged 7.0–11.2 months (14,23–26). In our retrospective study, consecutive patients treated with low-dose irinotecan were enrolled to minimize selection bias. Therefore, 37% of PS2-3 included in our study, while only 0–24% of PS2 and no PS3 patients were included in these studies. Actually, the median OS in patients with PS0-1 was 7.1 months in our study. The difference of patient characteristics between our study and previous studies might influence the results of OS although our results are needed to validate by prospective studies.

This study has several limitations. First, this is a retrospective study in a single institution. Efficacy evaluation schedules were not defined strictly due to the retrospective study from clinical practice. In addition, confirmation of CR/PR and external review of radiological imaging were not performed in this study. In regard to toxicities, the data were collected minutely from medical records and we could obtain each toxicity profile weekly due to the weekly schedule regimen; however, prospective comparative studies are needed to verify the less toxicity of low-dose irinotecan monotherapy.

In conclusion, low-dose irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) for recurrent SCLC was potentially active with a favorable toxicity profile. Further prospective trials are warranted to compare standard-dose irinotecan monotherapy versus low-dose irinotecan monotherapy for patients with recurrent SCLC.

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Conflict of interest statement

None declared.

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