ORIGINAL MANUSCRIPT

Expression and clinical significance of genes frequently mutated in small cell lung cancers defined by whole exome/RNA sequencing

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Abstract

Small cell lung cancer (SCLC) is the most aggressive type of lung cancer. Only 15% of SCLC patients survive beyond 2 years after diagnosis. Therefore, for the improvement of patients' outcome in this disease, it is necessary to identify genetic alterations applicable as therapeutic targets in SCLC cells. The purpose of this study is the identification of genes frequently mutated and expressed in SCLCs that will be targetable for therapy of SCLC patients. Exome sequencing was performed in 28 primary tumors and 16 metastatic tumors from 38 patients with SCLCs. Expression of mutant alleles was verified in 19 cases by RNA sequencing. *TP53*, *RB1* and *PTEN* were identified as being significantly mutated genes. Additional 36 genes were identified as being frequently (≥10%) mutated in SCLCs by combining the results of this study and two recent studies. Mutated alleles were expressed in 8 of the 36 genes, *TMEM132D*, *SPTA1*, *VPS13B*, *CSMD2*, *ANK2*, *ASTN1*, *ASPM* and *FBN3*. In particular, the *TMEM132D*, *SPTA1* and *VPS13B* genes were commonly mutated in both early and late stage tumors, primary tumors and metastases, and tumors before and after chemotherapy, as in the case of the *TP53* and *RB1* genes. Therefore, in addition to *TP53*, *RB1* and *PTEN*, *TMEM132D*, *SPTA1* and *VPS13B* could be also involved in SCLC development, with the products from their mutated alleles being potential therapeutic targets in SCLC patients.

Introduction

Small cell lung cancer (SCLC) is the most aggressive type of lung cancer with extremely poor prognosis (1–3). Therefore, it

is critical to develop novel therapies for the improvement of prognosis. Up to the present, extensive genome-wide molecular

analyses have been performed in SCLC (4–8). However, targetable genetic alterations have not been defined. This could be due to several reasons. First, mutation rates in SCLCs are very high. Second, except for the frequent inactivation of the *TP53* and *RB1* genes, the spectra of genes with high mutation frequencies are considerably different among studies. Third, only a small number of clinical SCLC cases have been analyzed to date, due to the small proportion of surgical cases. Fourth, no known druggable mutations have been identified to date. Therefore, we still do not have enough information to select genes with significant clinical importance and with druggable mutations in SCLC.

We previously performed the whole-genome copy number analysis and RNA sequencing of 58 and 42 SCLCs, respectively, and revealed the importance of amplification of *MYC* family genes and several other genes (8). In this study, we attempted to obtain further information on genes frequently mutated in SCLC through the analysis of 38 SCLC cases. The study confirmed high mutation rates in SCLC, and identified *TP53*, *RB1* and *PTEN* as significantly mutated genes in SCLCs. Furthermore, by a comparative study of our results with those of two previous studies (6,7), additional 36 genes were identified as being frequently (≥10%) mutated in SCLCs. Therefore, expression of mutated alleles in these genes was validated by whole transcriptome sequencing, and their mutation statuses were investigated in early and late stages of SCLCs, in primary tumors and metastases, and in tumors before and after chemotherapy. Mutated alleles were expressed only in 8 of the 36 genes, and sets of genes mutated in tumors were drastically different between early and late stages SCLCs, between primary tumors and metastases, and also between tumors before and after chemotherapy. We discussed the possible roles of significantly and frequently mutated genes in SCLC development, and defined several therapeutically targetable genes in SCLCs.

Patients and methods

Tissues

Forty-four tumors and the corresponding non-cancerous tissues were obtained from 38 patients with SCLC at surgery or autopsy from 1985 to 2010 at the National Cancer Center Hospital, Tokyo, Japan; Saitama Medical University, Saitama, Japan; University of Tsukuba, Ibaraki, Japan; and hospitals in the Metropolitan Baltimore area in the USA, and kept frozen until DNA and RNA extraction. Clinicopathological characteristics and sample information are summarized in Table 1 and Supplementary Table 1 is available at *Carcinogenesis* Online, respectively. The tumors were histologically diagnosed according to the 2004 WHO classification and pathologically staged according to the tumor-node metastasis classification of malignant tumors (9,10). Immunohistochemical staining data for neuroendocrine markers, synaptophysin, chromogranine and neural cell adhesion molecule (NCAM), were available in most cases; thus, were added in Supplementary Table 1, available at *Carcinogenesis* Online. Primary tumors were obtained from 25 cases, and metastases were obtained from 9 cases. Both primary tumors and metastases were obtained in cases 10 and 22. Two metastases were obtained in case 20, while the primary tumors and metastases to three different organs were obtained in case 21. Thirty cases were not treated by chemotherapy/radiotherapy, while the other 7 cases were treated before sampling. In case 10, the primary tumor was obtained before chemotherapy, and the metastasis was obtained after chemotherapy. Genomic DNA was extracted from frozen samples with a QIAamp DNA mini kit (Qiagen, Hilden, Germany). Total RNA was also extracted from frozen samples using TRIzol reagent (Invitrogen, Carlsbad, CA), purified by an RNeasy kit (Qiagen), and reverse-transcribed to cDNA by using the SuperScript III First-Strand Synthesis System (Invitrogen) with random hexamers. This study was performed under the approval of the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

Exome sequencing and Data processing

Methods of exome sequencing and data processing are summarized in Supplementary Figure 1 is available at *Carcinogenesis* Online. One microgram of genomic DNA was fragmented using the Covaris S220. After ligation of the paired end adapter including index, a fraction of 300–350 bp was gel-purified and amplified with PCR using the TruSeq DNA Sample Prep Kit (Illumina). Exome capture was performed using the TruSeq Exome Enrichment Kit (Illumina) according to the manufacturer's protocol. The resulting libraries were subjected to the paired end sequencing of 100-bp reads on the Genome Analyzer IIx (GAIIx) or HiSeq 2000 (Illumina). Mutation calling was performed using the EBCall algorithm (13). Germline variations represented in dbSNP Build 131 or 1000 Genome project (November 2010) and synonymous variations were filtered out. Variants present in the tumor with $P < 0.01$ by Fisher's exact test as compared to those in the matched normal tissue were predicted to be somatic mutations. Mean of target coverage in 44 tumors and 38 normal tissues was 97.3 (31.4–170.1) (Supplementary Figure 2, available at *Carcinogenesis* Online). MutSigCV analysis (14) was performed using expression profile data of 19 tumors analyzed by exome sequencing.

RNA sequencing and data processing

RNA sequencing was performed as described previously (8,15). Expression of mutated alleles detected by exome sequencing of the corresponding tumors was validated by a Bayesian inference method using the samtools and bcftools software (16,17).

Microarray experiments and data processing

Two micrograms of total RNA were labeled using a 5X MEGAscript T7 Kit (Ambion, Austin, TX) and analyzed by U133Plus2.0 arrays (Affymetrix). Data was processed by the MAS5 algorithm as described previously (18).

Results

Significantly mutated genes

Exome sequencing of 44 tumors from 38 SCLC patients identified 9279 protein-altering somatic mutations. In four cases with multiple tumors analyzed, common and unique mutations were detected between primary tumors and metastases and also between metastases of different organs. Therefore, total numbers of different somatic mutations among the multiple tumors in the same patients were considered as the number of somatic mutations in each case. Thirty-eight cases had an average of 244.2 protein-altering somatic mutations (19–1023) with the mean rate of 7.4 mutations (0.6–30.8) per megabase in

Table 1. Clinicopathological characteristics of 38 SCLC cases

Gender	
Male/female	28/10
Age	
Median (range)	67 (56–89)
Smoking status	
Ever/never	33/2 (3 unknown)
Brinkman index	
Median (range)	1000 (0–2040)
Pathological stage	
I/II/III/IV	10/8/10/10
Sampling at	
Surgery/autopsy	36/2
Treatment before sampling	
–/+	31/8[a]

[a]In one case, tumors were obtained before and after chemotherapy

5669 genes (Supplementary Figure 3, available at *Carcinogenesis* Online). Genes with protein altering mutations are listed with their nucleotide sequence changes in Supplementary Table 2, available at *Carcinogenesis* Online. G/C>T/A transversions occurred most frequently (Supplementary Figure 4, available at *Carcinogenesis* Online), consistent with the association of tobacco smoking with the occurrence of G/C>T/A transversions. Several software programs are now available to distinguish driver mutations from passenger mutations. We applied the MutSigCV method (14) to identify significantly mutated genes among the 5669 genes by integrating the expression profile data. Then, the *TP53*, *RB1* and *PTEN* genes were identified as being significantly mutated in SCLCs. *TP53* was mutated in 30 cases (78.9%), *RB1* in 28 cases (73.7%), and *PTEN* in five cases (13.2%) (Supplementary Table 3, available at *Carcinogenesis* Online). Types of *TP53*, *RB1* and *PTEN* mutations are summarized in Supplementary Table 4, available at *Carcinogenesis* Online. Previously, Peifer *et al.* (6). identified 22 significantly mutated genes, and Rudin *et al.* (7). identified another set of 22 significantly mutated genes in SCLCs. Although the criteria of 'significantly mutated gene' are different among three studies, both *TP53* and *RB1* were identified in all studies, while *PTEN* was identified only in this study (Supplementary Table 5, available at *Carcinogenesis* Online). *PTEN* was mutated in 2/27 (7.4%) and 2/30 (6.7%) in those studies, respectively (Supplementary Table 3, available at *Carcinogenesis* Online). Therefore, although *PTEN* mutations could be significant, its mutation frequency is much lower than those of *TP53* and *RB1* in SCLCs.

Frequently mutated genes

A total of 263 genes were mutated in ≥10% of the 38 cases (Figure 1). However, the power to detect frequently mutated genes could be small because of a small sample size in this study and a high background mutation frequency in SCLC. To increase the power of defining genes frequently mutated in SCLC, we further selected frequently mutated genes (≥10%) from two previous studies for whole exome sequencing of SCLC, in which the results of 27 and 30 SCLC cases were reported, respectively (6,7). Similarly high mutation rates of 7.4 and 5.5 per megabase, respectively, were reported in those studies, justifying a combined analysis. A total of 331 genes and 230 genes, respectively, were identified as being mutated in ≥10% of SCLC cases in those studies (Figure 1). Sets of frequently mutated genes were considerably different among three studies possibly

due to the difference in analytical methods used and the small number of cases analyzed in each study, and also due to the high mutation rates in SCLCs. SCLC samples were obtained mostly from Japanese in this study, while those were from Europeans/Americans in the previous studies. Therefore, it was also possible that such differences could be due to the ethnic and geographic differences of patients. Similarities in mutation frequencies and diversities in spectra of mutated genes among three studies indicated that genes commonly and frequently mutated in SCLC could be further selected by combining these data. Therefore, we further selected genes mutated in ≥10% of 95 SCLC cases, and also in ≥10% of 38 cases in this study and of 27 and 30 cases in two previous studies, respectively. A total of 38 genes were identified as being frequently mutated (≥10%) in all three studies as well as in a total of 95 cases (Figure 1 and Supplementary Table 3, available at *Carcinogenesis* Online). There was a low variability in the mutation frequencies of the 38 genes among three studies, and *TP53* and *RB1* were the first and second most frequently mutated genes in all studies. Thus, we concluded that, in addition to *TP53* and *RB1*, there are 36 genes that are frequently mutated in SCLCs irrespective of the geographic and ethnic differences of patients. *PTEN* was not included in the 38 genes, because of low frequencies of mutations (<10%) in two other studies.

Mutated genes with expression

MutSigCV analysis identified only three genes as being significantly mutated in SCLC. However, it is possible that there are additional genes whose mutations are also involved in SCLC development among the 36 genes frequently mutated in SCLCs. Indeed, four of the 36 genes, *TMEM132D*, *HCN1*, *SPHKAP* and *COL11A1*, were identified as being significantly mutated in SCLCs by Peifer *et al.* (6), and 2 of the 36 genes, *COL22A1* and *TMEM132D*, were also identified by Rudin *et al.* (7). Expression of the mutated allele is an important factor to support the significance of mutations in cancer development and to consider target therapies against mutated gene products. We previously performed whole transcriptome sequencing in 19 cases analyzed in this study (8). Therefore, transcripts from mutated alleles were searched for in the sequencing data of 36 frequently mutated genes in addition to *TP53*, *RB1* and *PTEN* (Figure 2). Expression of mutated alleles in the *TP53* and *RB1* genes was validated in several SCLC cases. *PTEN* was mutated only in one case, and expression of the mutated allele was not detected in this tumor. In addition, mutated alleles were expressed in 8 of the 36 genes, *TMEM132D*, *SPTA1*, *VPS13B*, *CSMD2*, *ANK2*, *ASTN1*, *ASPM* and *FBN3*, in at least one of the 19 tumors (Figure 2).

Mutated genes in stage I SCLCs before chemotherapy/radiotherapy

Driver mutations essential for SCLC development and its maintenance should be detected in early stage tumors before chemotherapy/radiotherapy. Therefore, we next analyzed the timing of mutation occurrence by considering the pathological stages and the effect of chemotherapy/radiotherapy. Average numbers of mutated genes tended to increase with stage progression (Supplementary Figure 5, available at *Carcinogenesis* Online), and significant difference in the number of mutated genes was observed between stage I tumors and stages II–IV tumors (*P* = 0.048 by the Student's *t*-test). The mean number of mutated genes in stage I tumors was 177.7, while that in stages II–IV tumors was 245.9 (Figure 3A). The result was consistent with the concept of sequential accumulation of somatic mutations during tumor progression (19–21). A total of 727 genes were mutated commonly in both stages I and II–IV tumors,
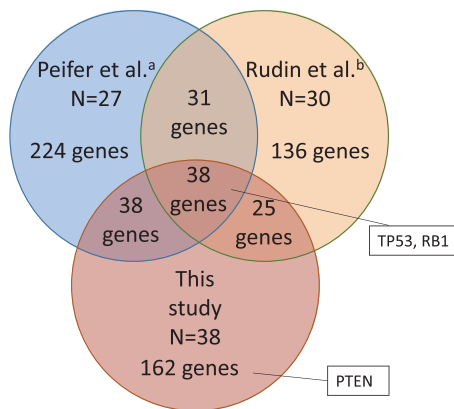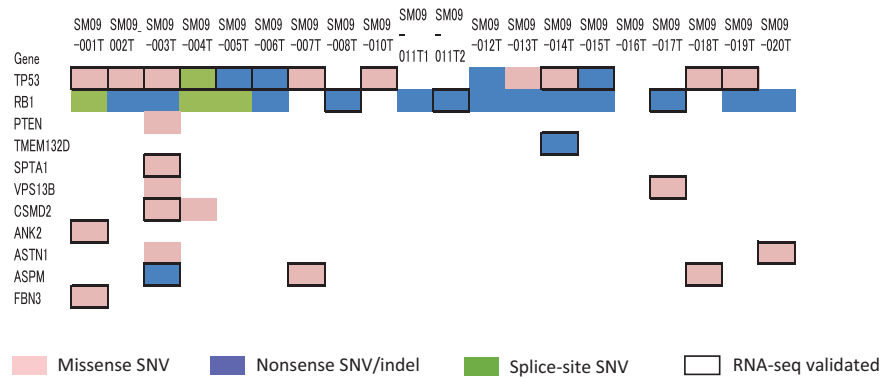

Figure 1. Numbers of genes frequently mutated in SCLC: Comparison between this study and two previous studies (a, ref 6; b, ref 7). Genes with mutation frequencies ≥10% of cases were selected in each study. Then, similarities and differences of genes selected were compared among three studies.

Figure 2. Expression of mutated alleles in significantly or frequently mutated genes in SCLC. Twenty tumors from 19 patients were analyzed by both exome sequencing and RNA sequencing. Types of mutations detected in tumors by exome sequencing are indicated by colored boxes, and expression of mutated alleles validated by RNA sequencing (RNA-seq validated) is indicated by black open squares.
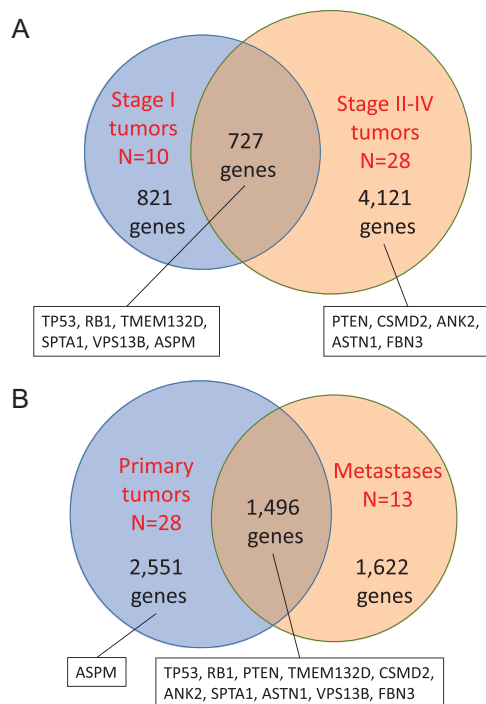

Figure 3. Number of genes mutated in early and late stages of SCLCs. (**A**) Comparison of mutated genes between stage I tumors and stage II–IV tumors. (**B**) Comparison of mutated genes between primary tumors and metastases. Mutation status of three significantly mutated genes and eight frequently mutated and expressed genes are indicated in the boxes.

while other 821 genes were mutated only in stage I tumors. It was noted that 4121 (72.7%) of the 5669 genes identified in this study were mutated only in stages II–IV tumors but not in stage I tumors. This result strongly indicates that most of mutations detected in SCLC cells have occurred during tumor progression, thus, may not be necessary for tumor development, although the small number of early stage tumors may influence this assumption. *TP53* and *RB1* were mutated in any progression stages of tumors, supporting their importance in tumor development and its maintenance. On the other hand, *PTEN* mutations were detected only in stages II–IV tumors. Among eight frequently mutated and expressed genes, four genes, *TMEM132D*, *SPTA1*, *VPS13B*, *ASPM*, were mutated in both stages I and II–IV tumors, while the remaining four genes, *CSMD2*, *ANK2*, *ASTN1*, *FBN3*, were mutated only in stages II–IV tumors but not in stage I tumors. Therefore, although the frequency of their

mutations were not significantly different between stage I tumors and stage II–IV tumors, the *TP53*, *RB1*, *TMEM132D*, *SPTA1*, *VPS13B* and *ASPM* genes could play more important roles in SCLC development than other genes.

We further investigated whether the 5669 genes were mutated before or after chemotherapy/radiotherapy (Supplementary Figure 6, available at *Carcinogenesis* Online). In case 10, mutation data in two tumors obtained before and after chemotherapy were analyzed separately. Importantly, 722 genes were mutated only in tumors after chemotherapy/radiotherapy, and 38 of them showed significantly higher mutation frequencies in tumors after therapy than in tumors before therapy (data not shown). Therefore, it is possible that these mutations have occurred after therapy. However, none of the eight genes frequently mutated and expressed in SCLCs and the *TP53*, *RB1* and *PTEN* genes showed higher mutation frequencies in tumors after therapy than in tumors before therapy. In particular, mutations in the *VPS13B* and *ASPM* genes were detected only in tumors before therapy and not in tumors after therapy. Thus, none of the 11 genes was considered to be secondary mutated after or during chemotherapy/radiotherapy.

Genes mutated in both primary tumors and metastases

Driver mutations essential for SCLC development and its maintenance should be detected in both primary tumors and metastases. Both primary tumors and metastases were analyzed in three cases; therefore, mutation data in primary tumors and metastases in these cases were analyzed separately. The mean number of mutated genes in 28 primary tumors was 194.1, while that in 13 metastases was 298.0. Although the difference was not statistically significant (*P* = 0.073 by the Student's *t*-test), metastases tended to carry more mutations than primary tumors (Figure 3B). This result was also consistent with the concept of sequential accumulation of somatic mutations during tumor progression (19–21). Although 1622 of the 5669 genes were mutated only in the metastases, 7 genes frequently mutated and expressed in SCLCs and the *TP53*, *RB1* and *PTEN* genes were commonly mutated in both primary tumors and metastases. In contrast, *ASPN* mutations were detected only in primary tumors but not in metastases suggesting their possibility of being passengers.

Genetic heterogeneity among multiple tumors in the same patients

We next compared the accumulated genetic alterations among multiple tumors in four cases. The results strongly support
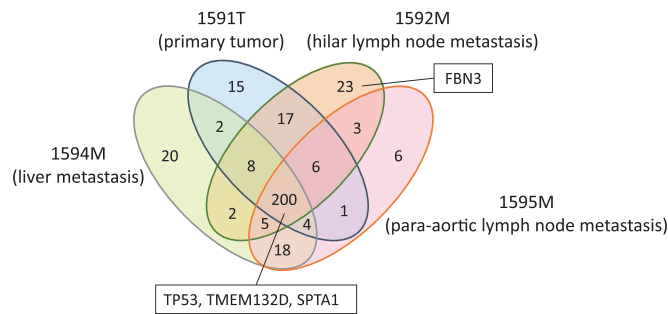
Figure 4. Heterogeneity in the accumulated mutations among multiple tumors in a single SCLC patient. The primary tumor (1591T), a hilar lymph node metastasis (1592M), a liver metastasis (1594M), and a para-aortic lymph node metastasis (1595M) from a single patient were analyzed for accumulated mutations by exome sequencing. Numbers in each region indicate the numbers of genes with mutations detected in tumors. Mutation status of one of significantly mutated gene, *TP53*, and three of frequently mutated and expressed genes, *TMEM132D*, *SPTA1* and *FBN3*, are indicated in the boxes.

the concept of clonal and parallel evolution of primary tumors and metastases (19–21). Namely, common and unique mutations were detected between primary tumors and metastases, and also between metastases of different organs (Figure 4; Supplementary Figure 7, available at *Carcinogenesis* Online). In particular, in the analysis of the primary tumor and three different metastases from a single patient, drastic heterogeneity in the accumulated mutations was observed among the four tumors (Figure 4). The *TP53*, *TMED132D* and *SPTA1* genes, which were defined as being frequently mutated and expressed in SCLCs, were mutated in all the tumors. In contrast, a mutation of another gene frequently mutated and expressed in SCLCs, *FBN3*, was detected only in a hilar lymph node metastasis but not in the primary tumor, a liver metastasis, and a para-aortic lymph node metastasis. Therefore, *FBN3* mutation could have occurred during tumor progression after *TP53*, *TMED132D* and *SPTA1* mutations.

Discussion

MutSigCV analysis revealed that *TP53*, *RB1* and *PTEN* were significantly mutated genes in SCLC. These genes are well-known tumor suppressors involved in the development of various cancers, including SCLC. Therefore, the present results further support that these genes are involved in SCLC development. However, this analysis failed to identify various other genes previously identified as being significantly mutated in SCLCs (6,7). Therefore, we further selected genes frequently mutated and expressed in SCLCs and investigated their clinical significance by analyzing their mutation status in various stages of SCLCs. As in the case of *TP53* and *RB1*, the *TMEM132D*, *SPTA1* and *VPS13B* genes were mutated in any stage of tumor progression and their mutated alleles were expressed in tumors. Recent comparative genomic analyses of primary and metastatic non-SCLCs and colorectal cancers revealed high concordance rates of driver alterations and low concordance rates of likely passenger alterations (22,23). Therefore, in addition to *TP53*, *RB1* and *PTEN*, *TMEM132D*, *SPTA1* and *VPS13B* could be also mutated as drivers in SCLC development. In particular, *TMED132D* was considered as being a significantly mutated gene in the two previous studies (6,7). Since mutated products from the *TMEM132D*, *SPTA1* and *VPS13B* genes are often expressed in SCLCs, those products could be novel therapeutic targets in SCLC patients.

In this study, *TP53* mutations were not detected in 8 of the 38 cases (21.2%), and *RB1* mutations were not detected in 10 of the 38 cases (26.3%). The results indicate the presence of a noble subset of SCLCs without *TP53* mutations and/or *RB1*

mutations. Therefore, it is very important to clarify whether the *TP53* and *RB1* genes are inactivated or not inactivated in tumors without detectable mutations by exome sequencing. We previously performed SNP array analysis in 19 of the 38 SCLC cases analyzed in this study (8). Therefore, the presence/absence of loss of heterozygosity (LOH) at the *TP53* and *RB1* loci were examined in tumors without *TP53* and/or *RB1* mutations (Supplementary Table 6, available at *Carcinogenesis* Online). LOH of the *TP53* locus was detected in 4 of 5 cases without *TP53* mutations, and LOH of the *RB1* locus was detected in 3 of 4 cases without *RB1* mutations. Therefore, it is likely that undetectable *TP53* and *RB1* mutations are present in the cases without their mutations. However, it is also possible that there is still a noble small subset of SCLCs without mutations of the *TP53* and/or *RB1* genes. Further molecular analyses, such as whole-genome sequencing and methylation array analysis in combination with mRNA expression array analysis, will be able to reveal the presence/absence of a noble subset of SCLCs.

Similar studies were also performed to predict whether the *TMEM132D, SPTA1* and *VPS13B* genes function as oncogenes or tumor suppressor genes. LOH, loss of wild-type allele as well as nonsense mutation were found in the *TMEM132D* gene, while missense mutations without allelic losses or with chromosomal gain were common in the *SPTA1* and *VPS13B* genes. Therefore, the *TMEM132D* gene is likely to function as a tumor suppressor gene, whereas the *SPTA1* and *VPS13B* genes are likely to function as oncogenes. However, since involvement of the *TMEM132D, SPTA1* and *VPS13B* genes in cancer development is unknown at present, functional studies will be necessary to elucidate the pathogenic significance of their mutations in SCLC development. The *TMEM132D* (transmembrane protein 132D) gene encodes a single-pass transmembrane protein, the *SPTA1* (spectrin, alpha, erythrocytic 1) gene encodes an actin crosslinking and molecular scaffold protein that links the plasma membrane to the actin cytoskeleton, spectrin, and the *VPS13B* (vascular protein sorting 13 homolog B) gene also encodes a potential transmembrane protein (NCBI databases: http://www.ncbi.nlm.nih.gov). The results indicate that mutated proteins are expressed on the membrane of SCLC cells. Therefore, although functional roles of these mutated gene products are presently unknown, the products could be appropriate targets of immunotherapy in SCLC patients.

Our study has several limitations. The number of cases analyzed in this study was small; therefore, we mainly analyzed the status of genes mutated in ≥10% of SCLCs. For the identification of targetable mutations in SCLCs, we should also analyze the status of genes with low frequency of mutations in SCLCs. As reported previously (6,7), we also found mutations in the *CREBBP*

(2 cases), EP300 (2 cases), Ephrin family (19 cases in 10 genes), and FLT family (7 cases in 3 genes) genes with low frequencies (<10%). Therefore, it is absolutely necessary to analyze a larger number of SCLCs to define the prevalence and clinical significance of their mutations. However, it is difficult to collect SCLC samples for molecular analysis, these results will be highly informative to further define genes targetable for therapy of SCLC patients.

Supplementary material

Supplementary Table 1–6 and Figures 1–7 can be found at http://carcin.oxfordjournals.org/

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