Clonal History and Genetic Predictors of Transformation Into Small-Cell Carcinomas From Lung Adenocarcinomas

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Purpose: Histologic transformation of EGFR mutant lung adenocarcinoma (LADC) into small-cell lung cancer (SCLC) has been described as one of the major resistant mechanisms for epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). However, the molecular pathogenesis is still unclear.

Methods: We investigated 21 patients with advanced EGFR-mutant LADCs that were transformed into EGFR TKI–resistant SCLCs. Among them, whole genome sequencing was applied for nine tumors acquired at various time points from four patients to reconstruct their clonal evolutionary history and to detect genetic predictors for small-cell transformation. The findings were validated by immunohistochemistry in 210 lung cancer tissues.

Results: We identified that EGFR TKI–resistant LADCs and SCLCs share a common clonal origin and undergo branched evolutionary trajectories. The clonal divergence of SCLC ancestors from the LADC cells occurred before the first EGFR TKI treatments, and the complete inactivation of both RB1 and TP53 were observed from the early LADC stages in sequenced tumors. We extended the findings by immunohistochemistry in the early-stage LADC tissues of 75 patients treated with EGFR TKIs; inactivation of both Rb and p53 was strikingly more frequent in the small-cell–transformed group than in the nontransformed group (82% vs. 3%; odds ratio, 131; 95% CI, 19.9 to 859). Among patients registered in a predefined cohort (n = 65), an EGFR mutant LADC that harbored completely inactivated Rb and p53 had a 43 × greater risk of small-cell transformation (relative risk, 42.8; 95% CI, 5.88 to 311). Branch-specific mutational signature analysis revealed that apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)–induced hypermutation was frequent in the branches toward small-cell transformation.

Conclusion: EGFR TKI–resistant SCLCs are branched out early from the LADC clones that harbor completely inactivated RB1 and TP53. The evaluation of RB1 and TP53 status in EGFR TKI–treated LADCs is informative in predicting small-cell transformation.

Introduction

Histologic transformation of EGFR mutant lung adenocarcinoma (LADC) into small-cell lung cancer (SCLC) has been identified as one of the major mechanisms of resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). Patients typically present with an aggressive clinical course after the transformation, which leads to a rapid deterioration of the patients’ condition. The treatment-resistant SCLCs seem to originate from preceding LADCs because they share the same type of EGFR mutations. However, the molecular pathogenesis of this process, especially when and how the LADC cells are transformed into SCLCs, remains elusive. A previous study indicated that the inactivation of RB1 is common in the transformed SCLCs but is not sufficient for LADC cells to transdifferentiate into SCLCs. To understand the transformation process, we studied patients whose advanced LADCs were transformed into SCLCs during EGFR TKI treatment and

Key points

- Histologic transformation of EGFR mutant lung adenocarcinoma (LADC) into small-cell lung cancer (SCLC) has been identified as one of the major mechanisms of resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs).
reconstructed their tumor clonal evolutionary history from whole genome sequencing (WGS). On the basis of these results, we examined the clinical implication of Rb and p53 expression status in early LADCs in predicting small-cell transformation.

### Methods

#### Study design

This study was approved by the institutional review boards of all participating centers. After approval, tissues were acquired with written informed consent. This study was conducted in observation of the most recent version of World Medical Association Declaration of Helsinki.

To identify patients with advanced LADC that transformed into SCLC, we first screened a cohort with advanced EGFR TKI–resistant SCLC who underwent repetitive biopsy at the point of EGFR TKI–resistance at the Seoul National University Hospital (n = 206). Second, we collected tissues from patients with advanced EGFR-mutant LADC that transformed into EGFR TKI–resistant SCLC at other institutions (n = 7 from Samsung Medical Center; n = 3 from Seoul National University Bundang Hospital; n = 1 from Boramae Medical Center).

DNA libraries for WGS were generated by a TruSeq PCR-Free Library Preparation Kit (Illumina, San Diego, CA) from 1 μg of genomic DNAs extracted from tumor tissues and matched normal blood samples.

We estimated tumor purity, ploidy, and allele-specific copy numbers from WGS data by using the Sequenza software package.

On the basis of this information, we calculated mutation copy number (n_{mut}) of high-confidence base substitutions from variant allele frequency information according to a previously described method. The number of chromosomal copies of each substitution (n_{chr}) was inferred from n_{mut} by selecting the closest positive integer value. Tumor cell fraction of each substitution was calculated as follows:

\[
\text{Tumor cell fraction} = \frac{n_{mut}}{n_{chr}}
\]

We reconstructed the phylogeny trees according to the tumor cell fraction of all the substitutions detected from serially acquired samples. The trees were validated by using the PhyloWGS software package.

#### Decomposition of mutational signatures

We decomposed mutational signatures from high-confidence base substitution sets by using the deconstructSigs software package. Eight mutational signatures previously identified and validated in lung cancers were used for analysis (signatures 1, 2, 4, 5, 6, 13, 15, and 17).

#### Pathologic examination

All pathology slides were examined by expert lung cancer pathologists (S.K., Y.K.J., and D.H.C.). The result of Rb immunohistochemistry (IHC) was classified as complete absence (no nuclear expression) or wild type (normal, variable nuclear expression). The result of p53 IHC was categorized as complete absence (no nuclear expression), overexpression (intense nuclear expression), or wild type (normal, variable nuclear expression), and complete absence of expression and overexpression were interpreted as evidence of p53 inactivation as described in previous studies.
**Results**

Clinicopathologic characteristics of 21 EGFR TKI–resistant transformed SCLCs

We recruited 21 patients with advanced LADC that was transformed into SCLC at the point of acquired resistance to EGFR TKI–treatment (defined by Jackman criteria\(^1\)). Among them, 10 patients (LC1 to LC10, which included one reported previously\(^1\) [LC1]) were from a single-institution cohort that comprised 206 patients with EGFR mutant–LADC who developed acquired resistance to EGFR TKIs. An additional 11 patients (LC11 to LC21) were identified from the medical oncology clinics of three different institutions (clinical history and pathologic findings of five patients were previously described in the literature\(^16,17\)). All 21 patients were initially given a diagnosis of an advanced LADC with canonical EGFR-activating mutation and underwent EGFR TKI–treatment to achieve a favorable response (Table 1). Among them, four (19%) had EGFR L858R mutation and six (29%) were male; both characteristics were rarely observed in the literature on small-cell transformation.\(^3\)

All but two patients (90%) were never-smokers or light smokers (< 10 pack-years). By using microscopic examination and IHC, we confirmed histology of all available serially acquired tumors.

Early branched ancestry of transformed SCLC from the LADC lineage

To explore the relationship between transformed SCLCs and their counterpart, early LADCs, we performed WGS of serially acquired tumors. These longitudinal sequencing analyses were available for four (LC1 to LC4) of the 21 patients. Overall, nine tumors (five SCLCs and their corresponding early LADCs; all fresh frozen except for two formalin-fixed paraffin-embedded early LADC tissues [LC2b and LC3a]) were sequenced for whole genomes, and their treatment history is summarized in Figure 1. In addition, we sequenced an SCLC tissue from a male light smoker that harbored an \(EGFR\) L858R mutation (LC0b), which was primarily resistant to gefitinib. We identified high-confidence somatic mutations, including base substitutions, short indels, and structural variations.

We first used the high-confidence somatic base substitutions to investigate the clonal relationship between paired tumors of each patient. Two-dimensional plotting of tumor cell fractions, the fraction of tumor cells that harbor each substitution, identified similar distributions with three mutational clusters in all patients (Figure 2A). The first cluster was observed at \([1.0, 1.0]\), which indicated truncal mutations were present in the most recent common ancestor cell of early LADC and transformed SCLC clones. The first clusters in two patients (Figure 2A) commonly had EGFR-activating mutations and inactivating mutations of \(RBI\) and \(TP53\). The second and the third clusters (present on the \(x\)- and \(y\)-axis, respectively) included late mutations on the branches, which were private for early LADCs and transformed SCLCs, respectively. Of note, this branched clonal relationship implies that the transformed SCLC clones did not originate directly from the major clones of preceding LADCs in all four patients. We further validated this exclusive clonal relationship by deeper sequencing of tumors from patient LC1 (LC1b and LC1c, exomes with approximately 150x coverage and targeted deep sequencing with approximately 20,000x coverage).

We further investigated the time point of clonal divergence. In patients LC1 and LC2, the early LADCs (LC1b and LC2b) were gefitinib-resistant–LADCs, and the transformed SCLCs (LC1c and LC2c) were diagnosed during subsequent treatments with third-generation EGFR TKIs. Of note, secondary \(EGFR\) alterations that conferred gefitinib resistance (ie, \(EGFR\) T790M\(^10,19\) and focal \(EGFR\) amplification) were only present in the gefitinib-resistant–LADCs (LC1b and LC2b) and not in their counterpart SCLCs (LC1c/d [Figure 2] and LC2c).

**Key points**

- We recruited 21 patients with advanced LADC that was transformed into SCLC at the point of acquired resistance to EGFR TKI–treatment (defined by Jackman criteria).
- All 21 patients were initially given a diagnosis of an advanced LADC with canonical EGFR-activating mutation and underwent EGFR TKI–treatment to achieve a favorable response.
- To explore the relationship between transformed SCLCs and their counterpart, early LADCs, we performed WGS of serially acquired tumors.
- We identified high-confidence somatic mutations, including base substitutions, short indels, and structural variations.
- We first used the high-confidence somatic base substitutions to investigate the clonal relationship between paired tumors of each patient.
- The first clusters in two patients commonly had EGFR-activating mutations and inactivating mutations of \(RBI\) and \(TP53\).
- The second and the third clusters (present on the \(x\)- and \(y\)-axis, respectively) included late mutations on the branches, which were private for early LADCs and transformed SCLCs, respectively.
- Of note, this branched clonal relationship implies that the transformed SCLC clones did not originate directly from the major clones of preceding LADCs in all four patients.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age*</th>
<th>Active smoking</th>
<th>Initial histology</th>
<th>Transformed histology</th>
<th>Neuroendocrine markers (C/S/N)</th>
<th>Treatment before transformation</th>
</tr>
</thead>
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<tr>
<td>LC1</td>
<td>F</td>
<td>56</td>
<td>Never</td>
<td>LADC</td>
<td>Del E746-A750</td>
<td>SCLC</td>
<td>+/-/+</td>
</tr>
<tr>
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<td>F</td>
<td>39</td>
<td>Never</td>
<td>LADC</td>
<td>Del E746-A750</td>
<td>SCLC with spindle cells</td>
<td>+/-/+</td>
</tr>
<tr>
<td>LC3</td>
<td>F</td>
<td>51</td>
<td>Ex-smoker, 0.5 pack-y</td>
<td>LADC</td>
<td>L858R</td>
<td>LADC + SCLC</td>
<td>+/-/+</td>
</tr>
<tr>
<td>LC4</td>
<td>F</td>
<td>39</td>
<td>Ex-smoker, 1.5 pack-y</td>
<td>LADC</td>
<td>Exon 19 del (not specified)</td>
<td>SCLC</td>
<td>+/---</td>
</tr>
<tr>
<td>LC5</td>
<td>M</td>
<td>50</td>
<td>Never</td>
<td>LADC</td>
<td>Del E746-T751 ins VP</td>
<td>SCLC</td>
<td>+/-/+</td>
</tr>
<tr>
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<td>79</td>
<td>Never</td>
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<td>Del E746-A750</td>
<td>LADC + SCLC</td>
<td>+/-/+</td>
</tr>
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<td>61</td>
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<td>SCLC</td>
<td>+/---</td>
</tr>
<tr>
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<td>L858R</td>
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<td>+/-/+</td>
</tr>
<tr>
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<td>F</td>
<td>58</td>
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<td>Del L747-P753</td>
<td>SCLC</td>
<td>+/---</td>
</tr>
<tr>
<td>LC10</td>
<td>M</td>
<td>62</td>
<td>Never</td>
<td>LADC</td>
<td>Del L747-T751</td>
<td>SCLC</td>
<td>+/---</td>
</tr>
<tr>
<td>LC11</td>
<td>F</td>
<td>54</td>
<td>Never</td>
<td>LADC</td>
<td>Del L747-A755 ins SRD</td>
<td>LADC + SCLC</td>
<td>ne/nehe</td>
</tr>
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<td>SCLC</td>
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</tr>
<tr>
<td>LC13</td>
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<td>59</td>
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<td>LADC</td>
<td>Exon 19 del (not specified)</td>
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<td>ne/ne/--</td>
</tr>
<tr>
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<td>37</td>
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<td>LADC</td>
<td>Exon 19 del (not specified)</td>
<td>SCLC</td>
<td>+/-/+</td>
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<tr>
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<td>ne/ne/+</td>
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<td>LADC</td>
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<td>SCLC</td>
<td>ne/ne/+</td>
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<td>Del E746-A750</td>
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<td>+/---</td>
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<td>LADC</td>
<td>L858R</td>
<td>SCLC</td>
<td>ne/+</td>
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<tr>
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<td>LADC</td>
<td>Del E746-A750</td>
<td>SCLC</td>
<td>ne/+</td>
</tr>
</tbody>
</table>

*Age at the diagnosis of stage IV disease.
C/S/N, chromogranin A/synaptophysin/neural cell adhesion molecule; EGFR, epidermal growth factor receptor; LADC, lung adenocarcinoma; ne, not examined; SCLC, small-cell lung cancer.
In contrast, some mutations, including \( \text{PIK3CA} \) mutations and \( \text{MYC} \) amplifications, were only observed in the transformed SCLCs, which implies that the third-generation \( \text{EGFR} \) TKI-resistant SCLC clones (major clones of LC1c and LC2c) were branched out before the acquisition of the focal \( \text{EGFR} \) amplification and/or T790M substitutions in their counterpart LADCs (major clones of LC1b and LC2b). This finding suggests that the clonal divergence likely preceded gefitinib treatment in both patients, although overt small-cell transformation appeared in the late stages (ie, 25 to 30 months after initial diagnosis). Furthermore, the branched clonal relationship in patient LC3 and patient LC4 (Figure 2) indicated that the branch out of SCLC ancestor clones even preceded the time points of initial diagnosis. In patient LC4, a retention of heterozygosity of chromosome 22 was observed in the transformed SCLC (LC4b), whereas a loss of heterozygosity was evident in early LADC masses.

**Key points**

- This finding suggests that the clonal divergence likely preceded gefitinib treatment in both patients, although overt small-cell transformation appeared in the late stages (ie, 25 to 30 months after initial diagnosis).
(LC4a; Figure 2B); this strongly supports the branched evolutionary relationship between LC4a and LC4b. Clonal comparison in patient LC3 was more challenging because of the low purity of LC3a and mixed histology of LC3b (LADC + SCLC); however, many high-confidence substitutions only present in LC3a (n = 453) also supported a branched clonal relationship between the paired tumors. We summarize the history of clonal evolution in four sequenced patients in Figure 3A.
Clonal History and Genetic Predictors of Transformation

**FIGURE 3** Phylogeny analysis of serially acquired tumors. (A) Phylogeny trees of sequenced patients (LC1 to LC4) are reconstructed from the whole genome sequencing data. Circles indicate major clones of the tumors. The length of each branch is proportional to the number of mutations that occurred in the branch. Mutations of cancer-related genes in each branch are indicated with arrows. The time points of relevant treatments are summarized beneath the trees. (B) Mutations of RB1 and TP53 in two early lung adenocarcinomas (LADCs; LC1b and LC4a) are visualized by using Integrative Genomics Viewer (left panel). Allele-specific copy number analysis shows loss of heterozygosity of chromosomes 13 and 17 in both early LADCs and epidermal growth factor receptor tyrosine kinase inhibitor–resistant small-cell lung cancers (SCLCs; right panel). (C) Clonal evolution of LC1 is described with clinical history and tumor volumes. The horizontal axis represents the time from the diagnosis (0), and the vertical axis indicates the volume of tumors calculated from the computed tomography images.

MRCA, most recent common ancestor.
To characterize the genomic instability process during small-cell transformation, we investigated mutational signatures.

- **Small-cell transformation is predictable by early complete inactivation of RB1 and TP53**

  We found that the biallelic inactivation of both RB1 and TP53 was observed from the early LADC stages in all the sequenced tumors except for one allele of TP53 in LC3a presumably because of the low purity or tumor heterogeneity of the sample.

  - At the molecular level, the inactivation of one parental copy of these genes was caused by diverse mechanisms, including frameshift indels, large deletions, and nonsense or inactivating missense substitutions. The other parental copy was invariably inactivated by whole chromosomal–or chromosomal arm–level loss-of-heterozygosity events in all four patients (Figure 3B). These biallelic inactivation events in both RB1 and TP53 were consistently shared by counterpart SCLCs in each patient. Although the biallelic, complete inactivation of the two canonical tumor suppressor genes are genetic hallmarks of the primary SCLCs, it is unusual for ordinary LADCs. Our reanalysis of The Cancer Genome Atlas data set indicated that 20 (5%) of the 416 patients with LADCs had mutations in both RB1 and TP53, the prevalence of which agrees with the observed prevalence of small-cell transformation (5%) in the current EGFR TKI–acquired resistance cohort in the literature. Therefore, we speculated that LADCs with the complete inactivation of both RB1 and TP53 are predisposed to transform into SCLCs. To elucidate this idea further, we evaluated the status of Rb and p53 by IHC in a wide range of 210 lung cancer tissues, including those that were small-cell transformed.

  First, we confirmed the expression of Rb and p53 in typical primary LADCs by using tissue arrays (n = 121; Figure 4A). Among 121 surgically resected LADCs, the inactivation of Rb and p53 was observed in five (4%) and 36 (30%), respectively. Overall, three tissues (2%) had concomitant inactivation of both Rb and p53. These data demonstrate the validity of IHC in assessing the status of Rb and p53 in LADC tissues.

  Next, we extended the IHC analysis to the initial EGFR mutant LADC tissues from patients with stage IV disease who received EGFR TKI treatment and ultimately developed a resistant tumor with a confirmed histology (Figure 4B). In a case-control comparison between the small-cell–transformed (n = 17) and nontransformed (n = 58) groups, we found that the complete inactivation of Rb and p53 was enriched among the initial LADCs of the small-cell–transformed group (14 of 17 vs. two of 58; odds ratio, 131; 95% CI, 19.9 to 859; Figure 4C). In three patients (18%), either gene was intact in the initial LADCs (n = 3) but subsequently inactivated in the transformed SCLC tissues. Except for the complete inactivation of both Rb and p53, we identified no other significant difference in clinicopathologic variables between the small-cell–transformed and nontransformed groups (Table 2). When we focus on the 65 patients (n = 7 small-cell–transformed; n = 58 nontransformed) in the predefined acquired resistance cohort from Seoul National University Hospital, LADCs with completely inactivated Rb and p53 had a 43× greater likelihood to transform into SCLCs during treatment (relative risk, 42.8; 95% CI, 5.88 to 311). These data demonstrate that complete inactivation of both RB1 and TP53 in LADC is a prerequisite for small-cell transformation and is detectable by IHC early during the LADC stage and, therefore, could be used as a strong predictive biomarker in clinics.

- **Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like–induced hypermutation in the branch of small-cell transformation**

  To characterize the genomic instability process during small-cell transformation, we investigated mutational signatures. The apolipoprotein B mRNA editing enzyme, catalytic polypeptidelike
FIGURE 4 Inactivation of Rb and p53 in early lung adenocarcinomas (LADCs) and their relationship with small-cell transformation. (A) Immunohistochemical staining of Rb and p53 in surgically resected early-stage LADCs. Representative images are shown (identification keys in the tissue array indicated beneath the images). Scale bar on the TA-8036 image indicates 50 μm, and all images were taken under ×400 magnification. (B) Expression status of Rb and p53 in tumor tissues of patients in the small-cell-transformed group (LC17, LC20, and LC21) and nontransformed group (CON16 and CON40). In the small-cell-transformed group, all tumors, including initial LADCs and transformed small-cell lung cancers (SCLCs), showed no expression of Rb. For p53, tumor pairs of LC17 and LC21 showed intense overexpression, whereas tumor pairs of LC20 showed complete absence of p53 expression. In contrast, the initial LADC tissues of CON16 and CON30 showed wild-type expression of Rb and p53. In CON16 and CON30, small-cell transformation was not observed during their clinical history, and their tumors became resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) by acquisition of EGFR T790M mutation. (C) Summary of the result of Rb and p53 immunohistochemical staining.
### TABLE 2 - Factors associated with histologic transformation into SCLC

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<th>Factor</th>
<th>No. of patients</th>
<th>Transformation into SCLC, no. (%)</th>
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<th>95% CI</th>
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<td></td>
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<td>&lt; 65</td>
<td>52</td>
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<td>≥ 65</td>
<td>23</td>
<td>2 (8.7)</td>
<td>0.235</td>
<td>0.0489 to 1.13</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>5 (20.0)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
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<td>50</td>
<td>12 (24.0)</td>
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<td>21</td>
<td>5 (23.8)</td>
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<td>EGFR mutation</td>
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<td>L858R</td>
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<td>13 (30.2)</td>
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<td>Use of third-generation EGFR TKIs*</td>
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<td>9 (25.7)</td>
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<td>40</td>
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<td>Time from diagnosis to last EGFR TKI failure, months†</td>
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<td>&lt; 30</td>
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<td>34</td>
<td>7 (20.6)</td>
<td>0.804</td>
<td>0.269 to 2.40</td>
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<td>Rb and p53 status in preceding LADC tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact either</td>
<td>59</td>
<td>3 (5.1)</td>
<td>Reference</td>
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<td>Both inactivated</td>
<td>16</td>
<td>14 (87.5)</td>
<td>131</td>
<td>19.9 to 859</td>
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</tbody>
</table>

Reference = 1.

*Third-generation EGFR TKIs included osimertinib, rociletinib, olmutinib, and EGF816.

†In all patients whose tumor underwent small-cell transformation, disease progression events to the last EGFR TKI treatment were attributable to small-cell transformation.

EGFR, epidermal growth factor receptor; LADC, lung adenocarcinoma; SCLC, small-cell lung cancer; TKI, tyrosine kinase inhibitor.
process can be hyperactivated during transformation into SCLCs.

Finally, we integrated tumor volume information, which was calculated from computed tomography images acquired through the treatment history of patient LC1 into our clonal analysis. As described in Figure 3C, the transformed SCLC and gefitinib-resistant LADC in patient LC1 underwent early branched and parallel evolutionary trajectories. The clonal divergence seemed to occur before the gefitinib treatment, and RB1 and TP53 were already inactivated at this point. A long latent period before the small-cell transformation, in which the APOBEC-induced hyper-mutation was operative, was apparent.

**Discussion**

Overall, this study indicates that EGFR TKI–resistant SCLC clones can be derived from divergent evolutionary processes from LADCs at the very early stage. Most importantly, this transformation process is already predisposed at the stage of early LADCs by complete inactivation of Rb and p53, which are predictive biomarkers for small-cell transformation.

Although the majority of transformed SCLC cases have been identified from EGFR-mutant LADC cases, studies have indicated that this phenomenon is not restricted to specific molecular subtypes; small-cell transformation could also occur in ALK-rearranged LADCs during ALK inhibition or at recurrence after surgical resection of a driver-unknown LADC. We also examined the expression of Rb and p53 in an initial LADC tissue without EGFR mutation from a previously reported patient whose tumor underwent transformation into SCLC during treatment. In the initial LADC as well as in the transformed SCLC, Rb and p53 were also completely inactivated. These findings suggest that small-cell transformation is more likely a clonal evolution event in LADC cells that is based on complete inactivation of RB1 and TP53. Therefore, an examination of the status of Rb and p53 in advanced LADCs merits consideration; LADCs with inactivation of these two genes may warrant observations for transformation into SCLC during their clinical history.

Multiplex genotyping of targetable cancer-related genes is becoming popular in medical oncology clinics, especially in those that treat patients with lung cancer. However, for tumor suppressors such as RB1 and TP53 in which the mutations are distributed along the entire length of a gene rather than concentrated in hot spots, the detection of all mutations sensitively by targeted deep sequencing is difficult. For example, RB1 is frequently disrupted by genomic rearrangements of which breakpoints are frequently located in introns; therefore, the detection of RB1 mutations sensitively by targeted deep sequencing assays is difficult. In this regard, we believe that the IHC for Rb is a reliable alternative assay for the RB1 mutation analysis. The IHC for p53 also showed a good correlation between mutational data in previous studies and the current study.

Our patients who underwent WGS showed favorable and prolonged response to EGFR TKIs. For example, patient LC1 exhibited −96.5% and −96.9% of tumor volume shrinkage in response to gefitinib and osimertinib, respectively, and those responses were durable for > 10 months. Patient LC4 also showed −95.9% of tumor volume reduction in response to osimertinib, which lasted for > 2 years. However, how the direct ancestor cells of transformed SCLCs, which were likely to exist as an LADC subclone before overt SCLC transformation.

**Key points**

- We integrated tumor volume information, which was calculated from computed tomography images acquired through the treatment history of patient LC1 into our clonal analysis.
- The IHC for p53 also showed a good correlation between mutational data in previous studies and the current study.
- Our patients who underwent WGS showed favorable and prolonged response to EGFR TKIs.
- Patient LC4 also showed −95.9% of tumor volume reduction in response to osimertinib, which lasted for > 2 years. However, how the direct ancestor cells of transformed SCLCs, which were likely to exist as an LADC subclone before overt SCLC transformation.
In either scenario, a few cancer cells should become dormant for a long period before overt clinical transformation. Of note, the number of C>T transitions in the CpG context (designated as signature 1, which originates from spontaneous deamination of methylcytosines), which are known to be proportional to the number of mitoses,33 among the transformed SCLC-private mutations was smaller than that among the LADC-private mutations in both patients LC1 and LC2, despite the SCLCs acquired at later time points than the LADCs. Thus, cell division time might be longer in the SCLC branch, which resembles the slow-growing properties of persister cells.31 During this stage, the APOBEC-induced hypermutation could occur in these latent cancer cells and is reminiscent of stress-induced hypermutation and related phenotypic switch in microbes during antimicrobial treatment, which maximizes their adaptability against selection pressure from the drugs.34 This study was initiated to understand the clonal history of EGFR TKI–resistant SCLCs transformed from LADCs by using longitudinal sequencing. Although a pattern of early branched evolution is suggested in all four patients, the small sample size does not exclude the possibility of other evolutionary trajectories (ie, linear evolution). For a more complete understanding, a similar study with a larger number of patients who undergo WGS may be necessary. In particular, the acquisition of high-quality paired tumor samples, including primary tumors, is challenging in clinic, and we have carefully included data from two formalin-fixed paraffin-embedded primary tumors and performed a conservative analysis for valid clonal comparisons that may be partly offset by the exclusion of true-positive mutations from the analysis.

This study highlights the common clonal dynamics and mutational processes that actively occur in LADC cells from the very early stage. An understanding of the patterns of cancer clonal evolution is essential for designing an optimal treatment strategy.

### Key points
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### References


