

Elucidation of radiation-resistant clones by a serial study of intratumor heterogeneity before and after stereotactic radiotherapy in lung cancer

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Abstract: Stereotactic radiotherapy (SRT) for inoperable stage I non-small cell lung cancer has shown promising results and is now an alternative therapy for this disease. Several reports have detailed changes in mutation profiles after treatment with chemotherapy; however, such changes after SRT for lung cancer have not been reported. A patient who received SRT for lung cancer developed local recurrence 9 months after treatment and underwent surgery in our department. Using bronchoscopically biopsied and surgically resected specimens, we performed targeted sequencing of 53 lung cancer-related genes and compared the tumor mutation profiles before and after SRT. Identical mutations were detected from tumor specimens collected before and after SRT, and the specimens were confirmed to be clonal. However, the number of mutations decreased after SRT, suggesting that it induced mutation selection. Analyses of the statistical inference of clonal population structure showed that this evolving heterogeneous genomic landscape may be caused by heterogeneous responsiveness to SRT.

Keywords: Stereotactic radiotherapy (SRT); mutation; lung cancer; next-generation sequencing

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Introduction

In recent years, stereotactic radiotherapy (SRT) has been effectively used to treat lung cancer, and it is steadily gaining acceptance as an alternative therapy to surgery for patients with physically inoperable conditions or those who refuse surgery (1-3). Meanwhile, there have also been sporadic reports of local recurrence and salvage surgery for local recurrence after SRT. Thus, the mechanism underlying tumor eradication by SRT warrants further investigation. In recent years, the detection of changes in mutation profiles after cancer treatment has attracted attention as a novel method for elucidating treatment efficacy (4). We encountered a patient who underwent surgery for local lung cancer recurrence after SRT. We investigated the therapeutic efficacy of SRT as a lung cancer treatment by

using molecular genetic testing to compare the lung cancer mutation profiles before and after SRT.

Case presentation

An 85-year-old woman visited our outpatient clinic for further examination of a right pulmonary nodule. Radiologic and bronchoscopic examinations revealed that the tumor was a cT1aN0M0 adenocarcinoma (*Figure 1A*). The patient refused surgery and chose to undergo SRT at a total radiation dose of 50 Gy in four fractions. Nine months after SRT, there appeared an area of consolidation that extended into the irradiated field (*Figure 1B*), and minimal pleural effusion also appeared in the right thorax. Radiologically, it was challenging to determine if the

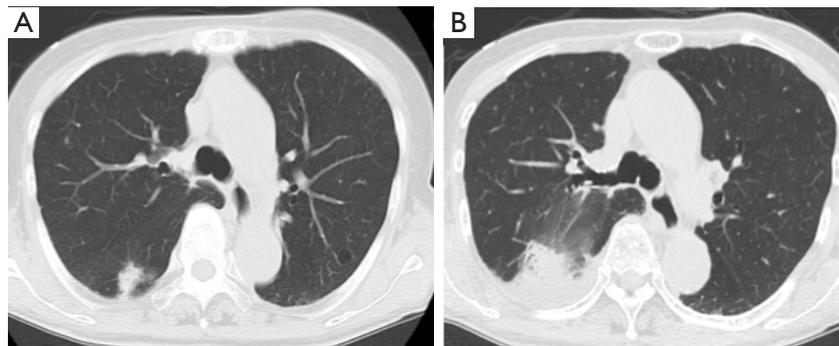


Figure 1 Radiologic findings of the lung cancer. (A) A chest computed tomography scan showed a mass that is 16 mm in diameter with an irregular surface in the right lower lobe; (B) 9 months after SRT, an area of consolidation and minimal pleural effusion appeared.

consolidated area was local tumor recurrence or prolonged inflammation due to the radiation (*Figure 1B*). However, because carcinoembryonic antigen (CEA) levels were persistently elevated, we suspected tumor recurrence. When we informed the patient of the suspected diagnosis, she requested surgery; thus, a right lower lobectomy and lymph node dissections were performed (*Figure 2A,B*). During surgery, cytology revealed malignant pleural effusion, so the operation was considered “incomplete salvage surgery”. Histopathologically, most of the resected tumor had been replaced by elastofibrosis, but the lepidic growth pattern of the adenocarcinoma was present at the periphery of the elastic lesion (*Figure 2C,D*). The tumor cells had invaded the visceral pleura, but no lymph node metastases were found pathologically. After surgery, serum CEA levels immediately declined to within the normal range (*Figure 3*). The patient was prescribed gefitinib, an epidermal growth factor receptor (EGFR)-targeted drug, and has been followed up at our outpatient clinic without any sign of recurrence to date.

Using a next-generation sequencer, we compared mutations in the specimens biopsied by bronchoscopic examination with those in the lung tumor surgical specimens. In both samples, cancer cells were collected from formalin-fixed paraffin-embedded tissues using laser capture microdissection. Tumor purity was about 80–90%. The total amount of DNA used for next-generation sequencing analysis was 1.62 ng of the biopsy specimens and 3.76 ng of the surgical specimens (*Table S1*). An assay system that could analyze the entire exon region of the 53 lung cancer-related genes (68,046 amino acids) was developed *in house*, and the samples were subjected to next-generation sequencing (5–7). This assay system was used to identify mutations in the 53 significantly mutated genes, which were

identified from 408 patients with lung cancer by total exon analysis (8,9). In our study, mutations with an allele fraction $\geq 20\%$ were deemed significant. Upon comparing mutations in the biopsy and surgical specimens, the same mutations in *EGFR* and *MGA* were detected, and interestingly, the positions and patterns of the base-pair substitutions for those mutations completely coincided (*Figure 4*). After SRT, the number of significant mutations decreased from three to two, and the number of insignificant mutations also decreased from eight to two (*Figure 4*).

To determine whether radiotherapy changed the tumor cell composition, we performed PyClone analysis, which estimates the fraction of cancer cells harboring a mutation (*Figure 5A,B*) (10). Using pairwise correlation of mutations with cellular prevalence, the identified mutations were classified into four distinct clusters (*Figure 5C*). Of note, the cellular prevalence of clones with *NOTCH1* mutations (p.Cys1374Tyr) was decreased after therapy (*Figure 5D*, cluster #2). In contrast, the cellular prevalence of tumor clones with *EGFR* and *MGA* mutations slightly increased after radiotherapy, indicating that these tumor clones remained after radiation therapy (*Figure 5D*, cluster #1). Together, these results suggest that radiosensitive or radioresistant tumor clones can coexist in a tumor (e.g., so-called intratumor heterogeneity); this heterogeneity may affect the response and resistance to radiotherapy.

This study was approved by the Institutional Ethics Board of Yamanashi Central Hospital, and the patient provided written informed consent to participate.

Discussion

In this study, we used genetic testing to investigate the therapeutic efficacy of SRT for lung cancer by comparing

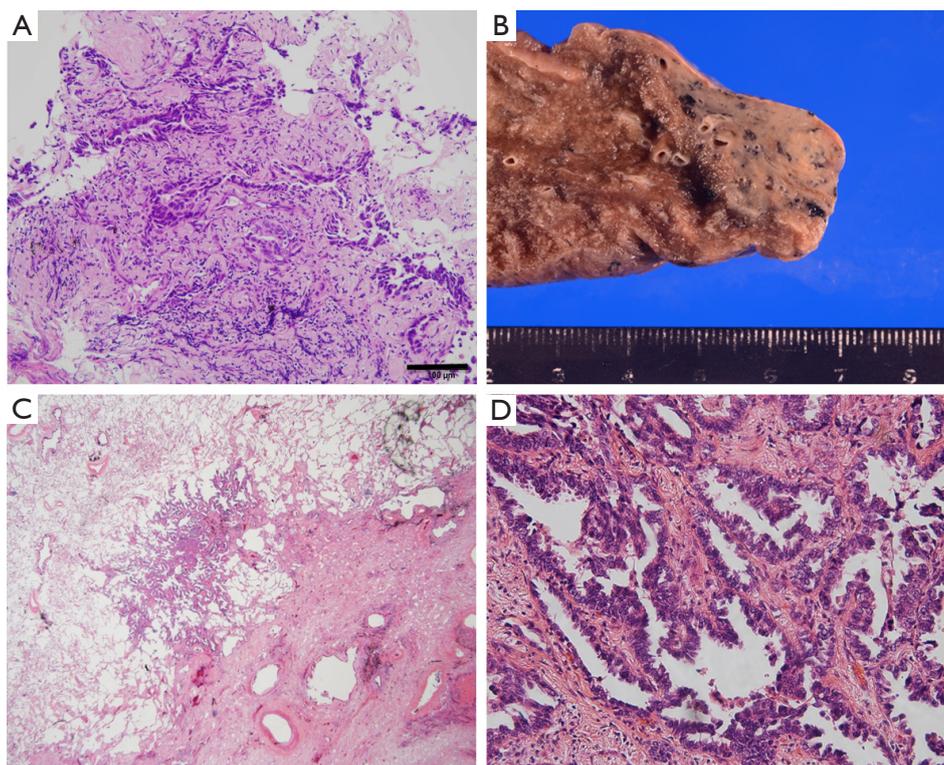


Figure 2 Pathological findings of the lung cancer. (A) A bronchoscopically biopsied specimen revealed invasive adenocarcinoma. The scale bar indicates 100 μ m; (B) macroscopically, the resected specimen appeared as a grayish-white tumor just adjacent to the visceral pleura; (C) the majority of the resected tumor histopathologically consisted of necrotic or fibrous tissue, but the lepidic growth pattern of adenocarcinoma was observed at the periphery of the necrotic lesion (hematoxylin and eosin staining, 40 \times , low-power field); (D) the tumor cells were hobnail-like and lined the alveolar septa, displaying a lepidic growth pattern (hematoxylin and eosin staining, 100 \times , high-power field).

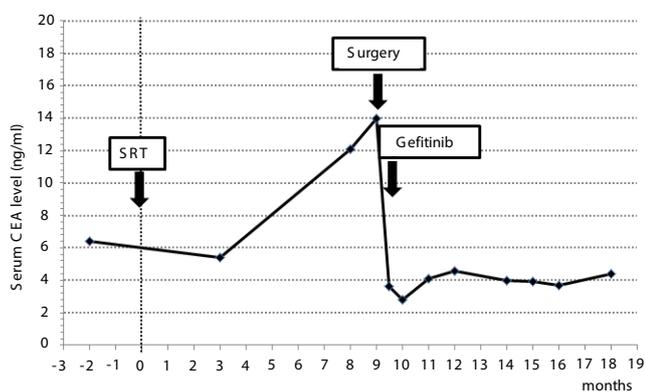


Figure 3 Time course of serum CEA level. After SRT, serum CEA levels elevated until we performed surgery. Serum CEA levels normalized after surgery and gefitinib administration. The time of SRT, surgery, and the onset of gefitinib administration are indicated by the arrows. The horizontal axis denotes time, and each scale on the axis is set at 1-month intervals.

mutation profiles between specimens obtained before and after treatment. Although local recurrence indicated cancer progression, the number of mutations decreased, demonstrating the therapeutic efficacy of SRT. The patient, who had stage IA adenocarcinoma at the time of her initial examination, refused surgery and underwent SRT. A number of phase 2 trials demonstrated promising results from SRT, and local control rates are reportedly 80–95%, similar to surgical series (1-3). Particularly in patients ≥ 80 years of age, similar to the patient in our case study, definitive SRT for early-stage non-small-cell lung cancer is efficacious and safe (11,12). Two independent, randomized phase 3 trials that assessed SRT in patients with operable stage I non-small cell lung cancer closed early due to slow accrual; however, it was demonstrated that SRT could be an option for treating operable stage I non-small cell lung cancer (13). Additional randomized studies comparing SRT

Sample	Gene	Protein	Position	Ref.	Variance	AF (%)
BF biopsy	<i>EGFR</i>	p.E746-T751insVA	chr7:55242478	GAATTAAGAGAAGCAACA	GTTGCT	37
BF biopsy	<i>NOTCH1</i>	p.Cys1374Tyr	chr9:139400227	C	T	35
BF biopsy	<i>MGA</i>	p.Ile2540Met	chr15:42054436	A	G	34
BF biopsy	FGFR1	p.Val638Met	chr8:38273423	C	T	7
BF biopsy	NOTCH1	p.His1032Tyr	chr9:139403399	G	A	5
BF biopsy	ARID1B	p.Gln156Glu	chr6:157099529	C	G	5
BF biopsy	KMT2D	p.Pro634Leu	chr12:49445565	G	A	5
BF biopsy	AKT2	p.Asp441Asn	chr19:40740997	C	T	4
BF biopsy	NOTCH2	p.Met2467Ile	chr1:120457944	C	T	4
BF biopsy	RBM10	p.Glu751Lys	chrX:47044559	G	A	4
BF biopsy	U2AF1	p.Arg119Cys	chr21:44514892	G	A	4
Surgery	<i>EGFR</i>	p.E746-T751insVA	chr7:55242478	GAATTAAGAGAAGCAACA	GTTGCT	50
Surgery	<i>MGA</i>	p.Ile2540Met	chr15:42054436	A	G	48
Surgery	SOX2	p.Gln296Ter	chr3:181431034	C	T	5
Surgery	<i>KMT2A</i>	p.Gln2537Glu	chr11:118374216	C	G	4

Figure 4 Mutation profiles of lung cancer before and after SRT. Mutations with an allele fraction $\geq 20\%$ are highlighted in red. Ref, reference; AF, allele fraction; BF, bronchoscopic fiber; SRT, stereotactic radiotherapy.

with surgery in patients with operable cancer are warranted.

In this case, we originally intended to perform salvage surgery; however, because pleural dissemination was detected, we were unable to perform complete curative surgery. In current clinical practice, radiological and pathological difficulties in differentiating between inflammation and local recurrence after SRT are problematic. In our case study, because the tumor was predominantly composed of elastic tissue, a differential diagnosis might have been difficult to make even if a biopsy had been performed (14-16). Therefore, the development of cancer-specific diagnostic techniques, such as genetic testing or use of genomic biomarkers, is needed.

In our study, *EGFR* and *MGA* mutations were identical in tumor specimens collected before and after SRT, suggesting that they belong to the same clone. Because of intratumor heterogeneity, tumors intrinsically contain various mutations (17,18). After exposure to radiation, it can be presumed that cancer cells with radiation-sensitive mutations are eradicated, and cancer cells with radiation-

resistant mutations survive.

PyClone is a Bayesian clustering method for grouping sets of deeply sequenced somatic mutations into putative clonal clusters, while estimating their cellular prevalence and accounting for allelic imbalances introduced by segmental copy number changes and normal cell contamination (10). Thus, PyClone infers accurate clustering of mutations that co-occur in individual cells (10). In our study, PyClone analysis suggested that cells harboring *NOTCH1* mutations were sensitive to radiation, whereas those harboring *EGFR* and *MGA* mutations were resistant to radiation. Recent studies suggest that activation of the *NOTCH* signaling pathway is related to radiation resistance in tumor cells (19-21). OncodriveROLE, a machine learning-based approach, indicated that the *NOTCH1* p.Cys1374Tyr mutation, as detected in our study, is a "loss-of-function" mutation (22,23). Thus, in our case, loss-of-function in the *NOTCH1* gene might have led to radiation sensitivity, resulting in the ability of cells harboring those mutations to be eradicated by radiotherapy.

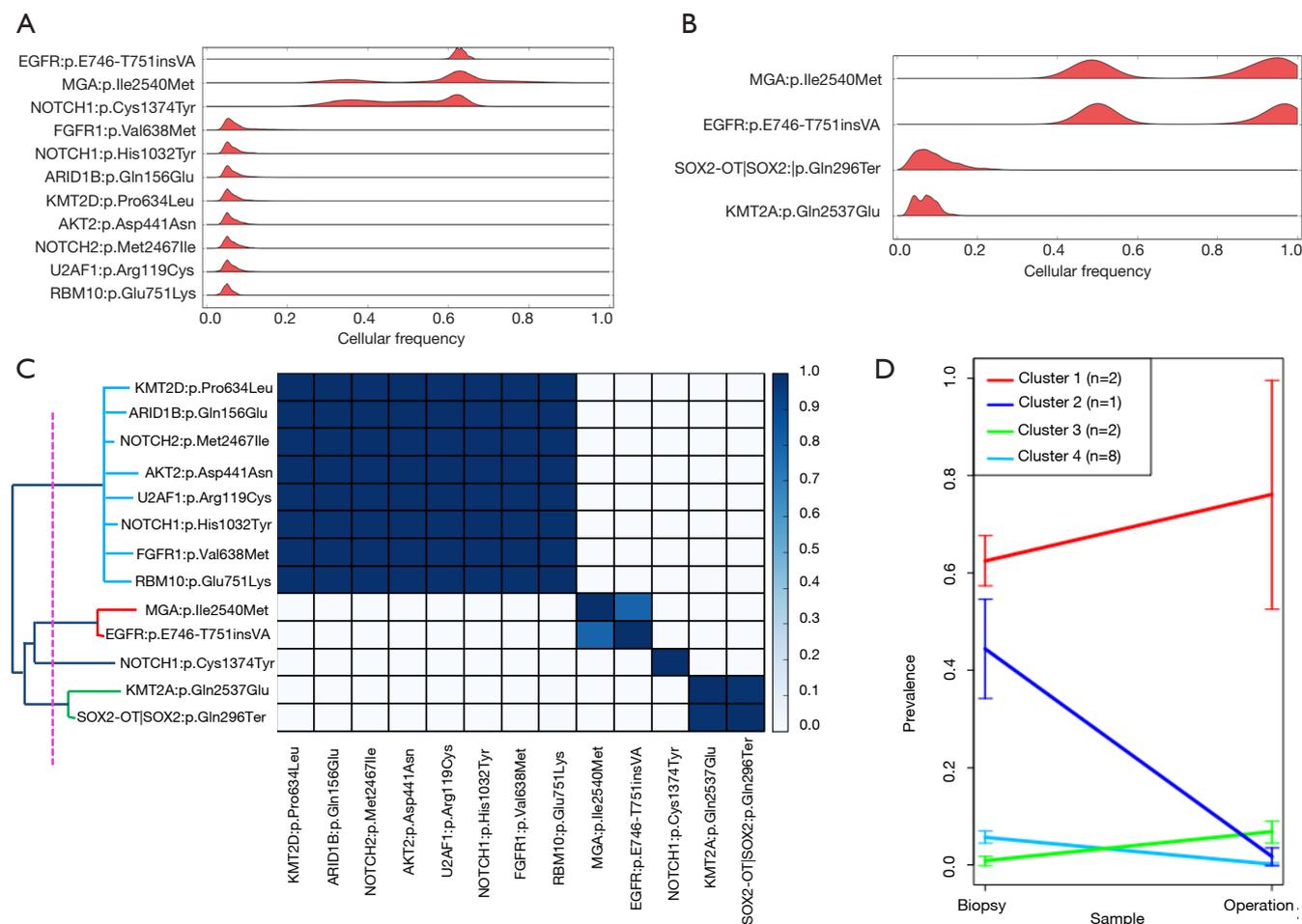


Figure 5 Statistical inference of the clonal population structure. (A,B) Estimated cellular frequencies of the samples before and after SRT are shown as the distribution of posterior probabilities from the PyClone model; sample A: pre-radiation biopsy, sample B: post-radiation resection; (C) the correlation between all possible pairs of mutations with cellular prevalence was assessed to identify mutations that share a similar pattern of cellular prevalence. Unsupervised hierarchical clustering was used to group correlated mutations into four clusters, with the dendrogram threshold level used to establish clusters indicated on the y-axis (dotted line); (D) the inferred cellular prevalence changed after SRT based on the following cluster configuration: cluster 1, EGFR and MGA; cluster 2, NOTCH1; cluster 3, KMT2A and SOX2-OT; cluster 4, other mutations.

We presume that this radiation-induced mutation selection resulted in decreased intratumor heterogeneity and a decreased number of detected mutations. Chemotherapy-induced changes in cancer mutation profiles have been reported, which can alter responsiveness to therapies (24-28). Here, we report that radiotherapy may also change lung cancer mutation profiles. However, because the broad clinical utility of our results has yet to be fully demonstrated, additional studies on similar cases treated with SRT are needed to validate our findings, as

well as to elucidate the genomic characteristics of radiation sensitivity and resistance. In addition, the development of new therapeutic strategies to eradicate patterns of mutation in cancer, such as a combination of molecular-targeted therapy and SRT, is needed.

In conclusion, genetically identical mutations were detected in tumor specimens collected before and after SRT, and those specimens were confirmed to arise from the same clone. Meanwhile, because the number of mutations decreased after SRT, we estimated that SRT induced

mutation selection. PyClone analysis showed that the therapeutic responsiveness of cancer to radiation varied among cells with different mutations, even in a single tumor. This condition is referred to as “heterogeneity in therapeutic responsiveness”, and may be responsible for the current difficulties in treating lung cancer.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Informed Consent: Written informed consent was obtained from the patient for publication of this manuscript and all accompanying images.

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Supplementary

Table S1 DNA isolation and analysis

Sample	Dissected area (mm ²)	Tumor purity (%)	Elution (μL)	DNA Concentration (ng/μL)	Amount of DNA used for NGS (ng)
Biopsy	15.7	80–90	30	0.27	1.62
Surgery	68.7	80–90	40	0.94	3.76

NGS, next generation sequencing.