ORIGINAL RESEARCH

Biopsy of palliative lesions following radiotherapy

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Objective: Definite radiotherapy and/or chemoradiotherapy is often conducted for the treatment of non-small cell lung cancer. However, there is a potential concern regarding the mutagenic effects on tumor cells derived from the therapies, and genomic information regarding cancer cells that survived definitive radiotherapy/chemoradiotherapy is lacking. To evaluate the mutagenic effect of radiotherapy/chemoradiotherapy, we compared genomic signatures of recurrent non-small cell lung cancer tissue with those of pre-treatment.

Methods: We evaluated seven specimens from three patients who developed disease recurrence after definite radiotherapy/chemoradiotherapy, and we ranked the mutations according to the Combined Annotation-Dependent Depletion score.

Results: Some mutations remained in the post-therapy state, and others, including driver mutations, either newly occurred or disappeared during the course of disease.

Of the four specimens obtained in the post-radiation period, 21 variants were detected. Compared with single nucleotide substitution (5, 23.8%), substantial number of deletions (16, 76.2%) was observed in specimens obtained after definite radiotherapy/chemoradiotherapy.

Conclusion: Radiotherapy/chemoradiotherapy effects on tumor cells have a wide spectrum, and resequencing of a recurrent lesion is always recommended to discuss the best course of therapy for recurrent non-small cell lung cancer after definitive radiotherapy/chemoradiotherapy.

Advances in knowledge: With regard to cancer cells that survived definitive radiotherapy/chemoradiotherapy, some mutations remained in the post-therapy state, and others, including driver mutations, either newly occurred or disappeared during the course of disease. Compared with single nucleotide substitution, substantial number of deletions was observed in specimens obtained after definite radiotherapy/chemoradiotherapy.

INTRODUCTION

Definitive thoracic radiotherapy for the treatment of non-small cell lung cancer (NSCLC) with curative intent is currently recommended in two settings: definitive radiotherapy with cytotoxic chemotherapy for locally advanced, inoperative disease and stereotactic thoracic radiotherapy for patients with node-negative early-stage disease who are not candidates for surgery.[1,2] In the case of disease recurrence, anticancer medications are considered the cornerstone of therapy. With the development of molecular-targeting therapy, patients are usually treated with molecular-targeting agents if a driver mutation is detected (*e.g. EGFR*).

Radiation is classified as ionizing radiation (IR: including X-rays, γ-rays, protons and carbon ions) and non-ionizing radiation. By making electrons free from atoms or molecules, IR induces chemical damage to DNA. The major effect of ionizing radiation on cancer cells is direct cell killing mostly by damaging DNA, resulting in the decrease in the cell numbers and subsequent tumor shrinkage.[3] Exposure to IR results in different types of DNA damage,

ranging from modified nucleotides to the most lethal form of damage, double-strand breaks.[4,5] Additionally, IR has indirect effects on cancer cells that have recently been proven to play a substantial role in radiation-induced mutagenesis.[5] By inducing the ionization and excitation of the water component of the cell, IR produces free radicals, which causes harmful effects on tumor cells.

There is a potential concern regarding IR-induced cytotoxic alterations on cancer cells; the alterations may result in the disturbance of tumor progression but may also affect the vital survival pathways of cancer cells. The mutagenic effects of IR on eukaryotic cells have been extensively studied. There is a possibility that driver genomic factors no longer drive tumorigenesis after chemoradiotherapy because of newly occurred tumor-maintaining genomic alterations induced by the mutagenic treatment. Currently, in the case of the recurrence of NSCLC, physicians decide on therapeutic agents based on the results of genomic analysis with specimens obtained at any time, usually before (chemo-)radiotherapy. There is little knowledge to make certain whether this any-time-is-OK strategy is adequate to

determine subsequent systemic therapy, and genomic information regarding cancer cells that survive definitive radiotherapy/chemoradiotherapy is lacking.

We compared genomic signatures of recurrent NSCLC tissue with those of pre-treatment to evaluate the mutagenic effect of radiotherapy/chemoradiotherapy on genes in NSCLC cells.

METHODS AND MATERIALS

Ethics
This study was approved by the local research ethics committee. All patients provided written, informed consent.

Biosamples and DNA isolations
All the biosamples used in the study were collected from a single institute. We used sections stained for hematoxylin and eosin to delineate cancerous components. The sections were used as blue prints to separately isolate cancerous components under microscopic control from deparaffinized serial sections. Tumor DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Germline DNA was isolated from peripheral blood mononuclear cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

Ion Torrent proton library preparation and sequencing
We used the Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific Inc, Waltham, MA), which covers the mutational status of 50 oncogenes and tumor-suppressor genes, for massively parallel panel sequencing (Table 1). An Ion Torrent adapter-ligated library was generated following the manufacturer's protocol (Thermo Fisher Scientific, Rev. A.0; MAN0006735). Briefly, 50 ng of pooled amplicons were end repaired, and Ion Torrent adapters P1 and A were ligated with DNA ligase. Following AMPure bead purification (Beckman Coulter, Inc., Brea, CA), the concentration and size of the library were determined using the Life Technologies StepOne™ system (Thermo Fisher Scientific Inc., Waltham, MA) and Ion Library TaqMan® Quantitation Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA).

Sample emulsion PCR, emulsion breaking, and enrichment were performed using the Ion PI™ Hi-Q™ Chef 200 Kit and Ion Chef™ (both from Thermo Fisher Scientific, Inc., Waltham, MA). Template-positive ISPs were enriched, and sequencing was performed using Ion PI Chip v3 chips on the Ion Torrent Proton and barcoding was performed using the Ion DNA Barcoding kit (Thermo Fisher Scientific, Inc., Waltham, MA).

Variant calling
Data runs were initially processed using the Ion Torrent platform-specific pipeline software, Torrent Suite, to generate sequence reads, trim adapter sequences, filter, and remove poor signal-profile reads. Initial variant calling from the Ion AmpliSeq sequencing data was generated using Ion Torrent cloud-based pipeline Ion Reporter v5.0. We used cut-off values greater than 10% of the variant frequency and more than ×200 coverage to detect true variants in accordance with previous reports and our own experience. To eliminate erroneous base calling, the second filter was employed by visually examining the mutations using CLC Genomics Workbench version 9.5.1 (Qiagen, Hilden, Germany), as well as by filtering out possible strand-specific errors.

Combined annotation-dependent depletion (CADD) scores
A general framework CADD was developed to establish a framework for variant-effect estimation, by integrating diverse genome annotations and scoring any possible human SNVs or small indels.[6] We obtained a CADD score for each identified variant via a web server,[7] and those with a high CADD score (≥20) were considered deleterious.
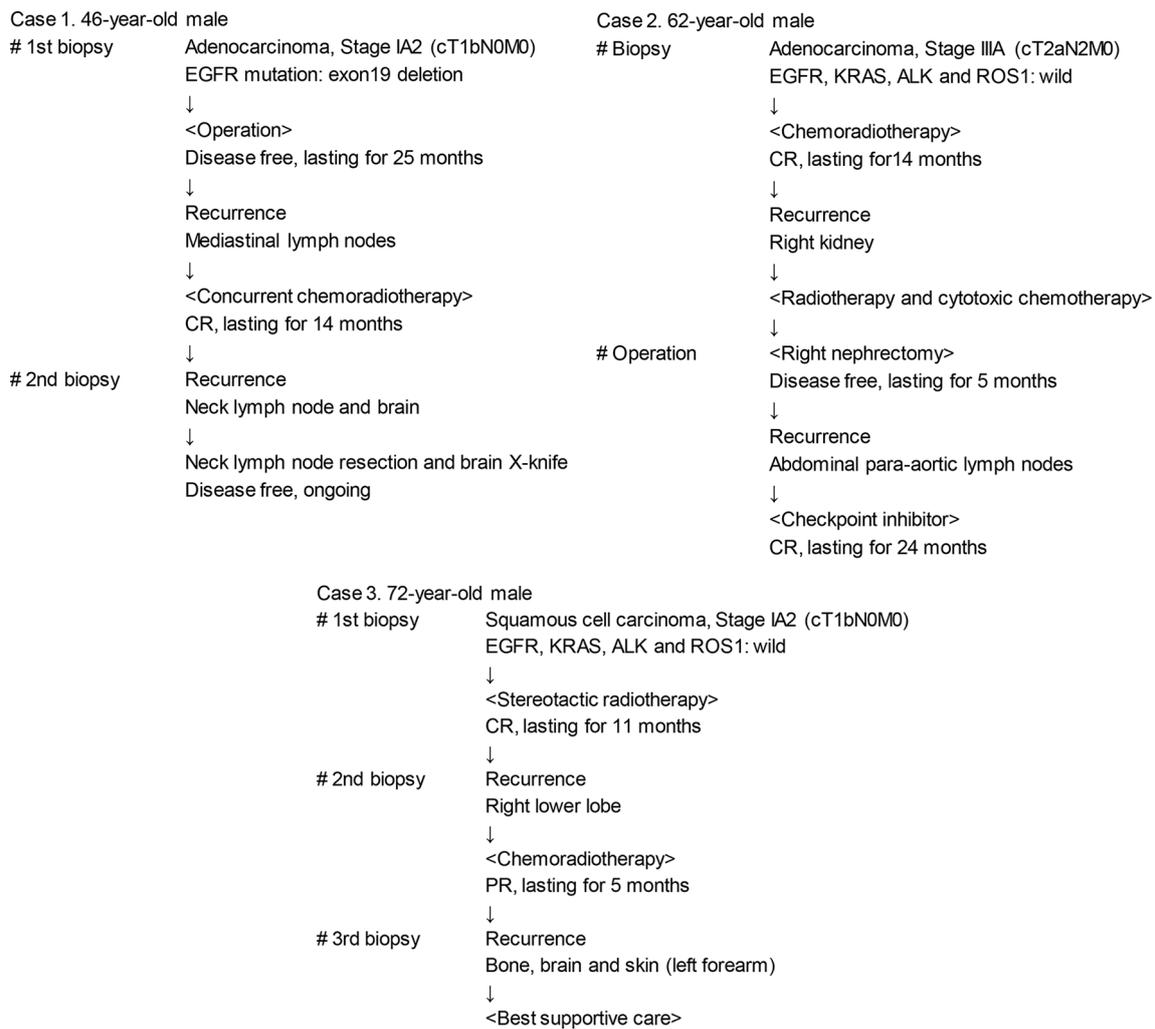
RESULTS
Four patients were enrolled in the study. However, we could not evaluate the pre-treatment specimen of one patient due to inadequate tissue quantity for genomic evaluation. The characteristics and clinical course of the three evaluated patients are shown in Figures 1 and 2. Pre-treatment assessments included a CT scan of the thorax and abdomen, a PET-CT scan and an MRI of the brain. All patients experienced complete remission or a disease-free status after first-line therapy. The tumor specimen from Case 1 was proven to be driver mutation positive (*EGFR* exon 19 deletion) at the initial diagnosis. The remaining patients were at first considered to be driver mutation negative (*EGFR*, *KRAS*, *ALK*

Table 1. The Ion AmpliSeq Cancer Hotspot Panel v2 targets the 50 genes described in the table

ABL1	EGFR	GNAS	KRAS	PTPN11
AKT1	ERBB2	GNAQ	MET	RB1
ALK	ERBB4	HNF1A	MLH1	RET
APC	EZH2	HRAS	MPL	SMAD4
ATM	FBXW7	IDH1	NOTCH1	SMARCB1
BRAF	FGFR1	JAK2	NPM1	SMO
CDH1	FGFR2	JAK3	NRAS	SRC
CDKN2A	FGFR3	IDH2	PDGFRA	STK11
CSF1R	FLT3	KDR	PIK3CA	TP53
CTNNB1	GNA11	KIT	PTEN	VHL

Figure 1. Clinical course of each enrolled patient. The timing of the biopsies is shown in the figures.

Case 1. 46-year-old male

# 1st biopsy	Adenocarcinoma, Stage IA2 (cT1bN0M0)
	EGFR mutation: exon19 deletion
	↓
	<Operation>
	Disease free, lasting for 25 months
	↓
	Recurrence
	Mediastinal lymph nodes
	↓
	<Concurrent chemoradiotherapy>
	CR, lasting for 14 months
	↓
# 2nd biopsy	Recurrence
	Neck lymph node and brain
	↓
	Neck lymph node resection and brain X-knife
	Disease free, ongoing

Case 2. 62-year-old male

# Biopsy	Adenocarcinoma, Stage IIIA (cT2aN2M0)
	EGFR, KRAS, ALK and ROS1: wild
	↓
	<Chemoradiotherapy>
	CR, lasting for14 months
	↓
	Recurrence
	Right kidney
	↓
	<Radiotherapy and cytotoxic chemotherapy>
	↓
# Operation	<Right nephrectomy>
	Disease free, lasting for 5 months
	↓
	Recurrence
	Abdominal para-aortic lymph nodes
	↓
	<Checkpoint inhibitor>
	CR, lasting for 24 months

Case 3. 72-year-old male

# 1st biopsy	Squamous cell carcinoma, Stage IA2 (cT1bN0M0)
	EGFR, KRAS, ALK and ROS1: wild
	↓
	<Stereotactic radiotherapy>
	CR, lasting for 11 months
	↓
# 2nd biopsy	Recurrence
	Right lower lobe
	↓
	<Chemoradiotherapy>
	PR, lasting for 5 months
	↓
# 3rd biopsy	Recurrence
	Bone, brain and skin (left forearm)
	↓
	<Best supportive care>

fusion and *ROS1* fusion gene). All the patients had a history of smoking.

Regarding the clinical course of Case 1, concurrent chemoradiotherapy was administered with curative intent at the time of the first relapse (mediastinal lymph node involvement). The recurrent right kidney tumor was treated with radiotherapy and cytotoxic chemotherapy (cisplatin and vinorelbine) in Case 2. Despite multimodality treatment, the tumor was enlarged to the level where serious pain annoyed the patient. Salvage right nephrectomy had been conducted, and DNA from the resected tumor was analyzed in the study. Biopsy was conducted three times on the lesions of Case 3. The third biopsy was performed on the skin lesion in the left forearm.

The mutation spectra of the patients are shown as Venn's diagram (Figure 2). Numerical scores under the gene names represent CADD scores. Some mutations persisted in the post-radiation period, and other mutations either newly occurred or disappeared during the course of the disease. From the four specimens obtained in the study, we identified 83 variants; 54 (65.1%)

originated from single-nucleotide substitution, 23 (27.7%) from small deletions, 5 (6.0%) from small insertions and 1 (1.2%) from base replacement (cytosine with tandem thymine; C with TT). Of the four specimens obtained in the post-radiation period (in Case 3, two biopsy specimens), 21 variants were detected. Compared with single-nucleotide substitution (5, 23.8%), a substantial number of deletions (16, 76.2%) was observed in specimens obtained after radiotherapy. As a result, the loss-of-function effect due to frameshift mutation was broadly observed.

In Case 1, activating *EGFR* mutation was retained after concurrent chemoradiotherapy, and additional deleterious mutations occurred. In the post-radiation specimen of Case 2, there was a common *KRAS* G12C activating mutation (allelic frequency of 49.5%) that had been observed with a low percentage (1.7%) of the pre-treatment biopsy specimen. Besides *EGFR* and *KRAS*, four genes (*ABL1*, *FGFR1*, *MET* and *KIT*) are classified as proto-oncogenes; however, in these four genes, we did not find any of the previously reported gain-of-function mutations. The remaining mutated gene with a high CADD score has a tumor-suppressor

Figure 2. Venn's diagram represents the mutations observed in cancer tissues. In each circle, the names of the genes mutated in the cancer specimens, along with the CADD scores obtained via the web server, are shown. CADD,combined annotation-dependent depletion.
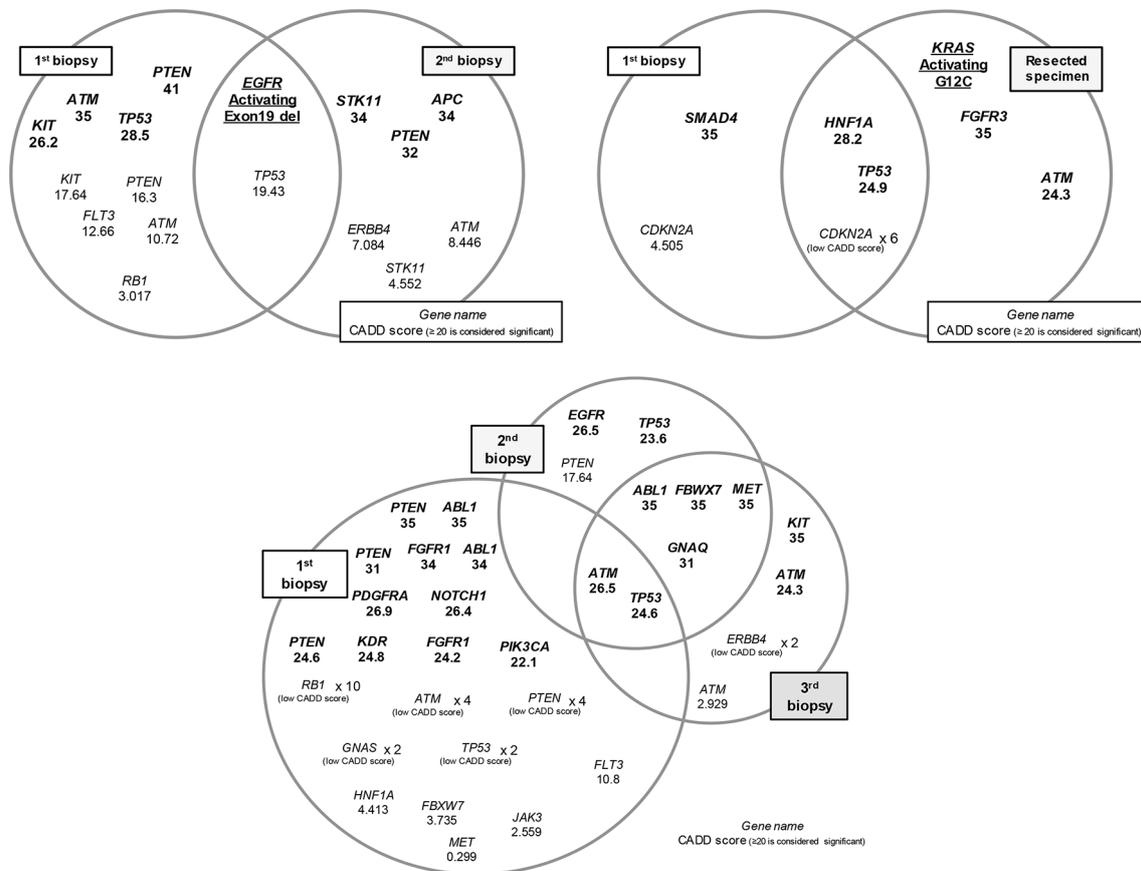
effect, and the observed mutations of the genes are predicted to be deleterious.

DISCUSSION

We compared the mutation spectrum from the specimens obtained post- (chemo-)radiotherapy to those from pre-treatment specimens in the study, and a substantial number of deletions was observed. This finding is compatible with those of previous reports. Adewoye and colleagues reported the results of a genomewide survey of germline mutations induced in mice after parental exposure to ionizing radiation.[4] They showed that the frequency of *de novo* copy number variants and ins/del is significantly elevated in the offspring of exposed fathers. Behjati and colleagues reported the mutational signatures of 12 human cancers that arose from previously irradiated sites.[8] They noted that the ins/del burden was high compared with the nucleotide substitution burden of the tumor. They also noted a significant excess of deletions relative to insertions in radiation-associated second malignancies. Because most of the mutations observed in human malignancies are single-nucleotide substitutions, the high percentage of deletions (16, 76.2%) found after (chemo-)radiotherapy in our study is unusual and the observation at least deserves further verification in the era of immune-oncology. Tumor mutation burden is demonstrated to be a useful biomarker

for immune checkpoint blockage selection across some cancer types including NSCLC.[9,10] Ins/del variants are more likely to contribute to increase of mutation burden compared to single nucleotide variants, because ins/del variants in coding exons almost always result in non-synonymous alterations.

In the study, the ratio of the mutated *KRAS* allele rose from 1.7 to 49.5% after chemoradiotherapy in Case 3. *KRAS* is one of the most mutated genes in human cancers, and approximately 15–25% of NSCLC tumor cells harbor a driver mutation of the gene.[11] Several hotspot mutations (*e.g.* G12X or G13X) are known to confer tumorigenesis, and a recent study suggested that the *KRAS* driver mutations are rather clonal (present in all cancer cells) in lung adenocarcinoma and occur in early stage of the tumor evolutional process, contrary to the result of our study. However, a recent study reported that a serial change of mutant/wild *KRAS* allele ratio was observed during progression and/or chemotherapeutic treatment in cancer cells. Burgess et al reported that *KRAS* G12D driver-mutation-positive acute myeloid leukemia cells in a mouse model responded to MEK inhibitors and that secondary alteration of the mutant *KRAS* copy number contributes to the resistance to these inhibitors.[12] In the same model, frequent *KRAS* G12D copy number gain and loss of wild-type *KRAS* allele in primary cancer cells were observed.

They also reported that 642 of 1168 cases of human lung cancer with *KRAS* mutation exhibited allelic imbalance; tumor cells do not contain one normal and one mutant allele. Moreover, *KRAS* driver mutations are defined as late events (*e.g.* subclonal) in the evolutional process of lung squamous cell carcinoma.[13] These observations indicate that the change in the *KRAS* mutated allele frequency is a dynamic process, and we should be cautious in evaluating the *KRAS* status of the tumors.

Strengths of our study include germline-based annotation and mutation-effect estimation by CADD scoring. A recent analysis suggested that a tumor-only sequencing approach led to false-positive findings comprising 31% of alterations identified in target analyses, and the current guideline recommends the usage of germline genome information to evaluate somatic mutation of tumors.[14,15] With CADD, we obtained a framework to integrate information contained in driver annotations of genetic variants into a single score.

There are several limitations in the study. First, we could only conduct panel-based NGS sequencing of a small number of lesions due to the poor condition of the small, time-elapsed formalin-fixed paraffin-embedded biopsy specimens. Several computational methods that identify the number and genetic composition of subclones by analyzing the variant allele frequencies have been developed, however, these methods require information regarding variant allele frequencies of several hundred loci across whole chromosomes, which are unavailable with the panel used in this study. Next-generation sequencing has become a powerful and widely used clinical tool to evaluate driver and passenger mutations in cancer, and there has been a worldwide trend to preserve well-conditioned tissue for future intensive genomic analysis. Exome-based or whole-genome-based clinical sequencing may become popular with abundant specimens. Second, the CADD algorithm has, like other *in silico* function-predicting programs, inadequate accuracy regarding the

gain-of-function change in genes, and we considered the gain-of-function when the observed mutations were biologically proven previously and were recorded in a public archive.[15] There are no ideal computational programs or tools to estimate the *de novo* gain-of-function mutation precisely. Third, we are unable to rule out the possibility that evolutional change in cancer genome is the only cause of the mutational signature observed in our study and that this mutation pattern is eventually observed, whether previous treatment includes radiotherapy or not. This pattern is neither tobacco-related (C > A mutations predominate) nor due to over activity of APOBEC (C > T and C > G mutations at TpCpN trinucleotides) and further case-controlled, comprehensive (genome-wide or exome-wide) study is awaited.[16]

We conclude that radiotherapy/chemoradiotherapy effects on tumor cells have a wide spectrum, and resequencing of a recurrent lesion is always recommended to discuss the best course of therapy for recurrent NSCLC after definitive radiotherapy/chemoradiotherapy, especially because the current progress regarding NGS technology enables researchers to study comprehensive genomic alterations of tumors rapidly and with low cost.

ETHICS APPROVAL
This study was approved by the local research ethics committee.

CONSENT
All patients provided written, informed consent.

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