

Table 1 Patient characteristics

	Total	Breast/prostate	Other
Number of patients	54	26	28
Age, median (range) (years)	64 (34–89)		
Gender, male/female	25/29	12/14	13/15
PS, 0–1/2/3	22/23/9	14/9/3	8/14/6
Primary site			
Breast		15	
Prostate		11	
Lung			8
Head and neck			6
Colorectal			6
Other			8

PS performance status

Table 2 Toxicity

	Grade (CTCAE ver. 4.0)		
	3	4	% 3/4
Leucopenia	0	1	1.8
Neutropenia	0	1	1.8
Anemia	5	1	11.1
Thrombocytopenia	2	2	7.4

CTCAE common terminology criteria for adverse events

continued to receive bisphosphonates during and after Sr-89 administration. Sixteen (70 %) of the breast/prostate cancer patients received bisphosphonate therapy, although only 7 patients (25 %) of patients with other cancers received therapy.

Toxicity

Thirteen (24 %) patients experienced a transient increase in pain, which was classified as a flare-up response. Profiling of other nonhematological toxicities was hampered by the frequent use of additional supportive interventions during and after protocol treatment, including morphine or other medications. Hematological toxicity is summarized in Table 2. Grade 3–4 anemia was observed in 6 patients, 3 of whom required blood transfusion within 2 months after protocol treatment. One patient developed disseminated intravascular coagulation (DIC), which might have been related to either Sr-89 administration or primary disease progression or to both.

Efficacy

Two patients were excluded from response evaluation because of sudden death unrelated to the use of Sr-89

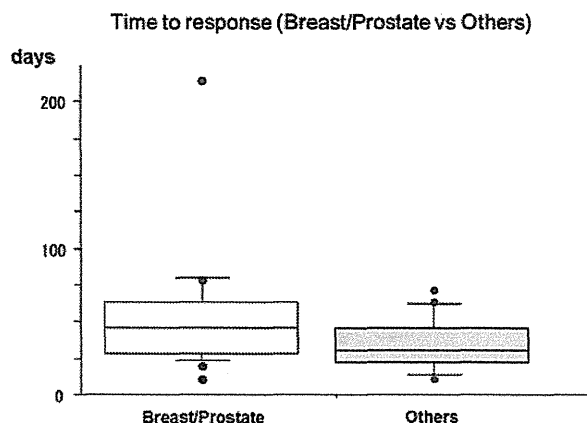


Fig. 1 Time to response (breast/prostate cancer vs. other). Time to response was calculated from the initiation of treatment to the day of pain relief (\geq PR). Median time to response in breast/prostate cancer patients was 46 days (range, 13–217); that in other cancer patients was 31 days (range, 14–73). There was no significant difference between breast/prostate cancer patients and others

chloride. One patient with lung cancer died 7 days after Sr-89 administration. Hepatic failure caused by liver metastases was considered to be the main cause of death. Another patient with gastric cancer died 31 days after Sr-89 administration. At first visit after Sr-89, his performance status was 2. However, after the first visit, his condition was rapidly worsened by cachexia.

Overall response rate was 71.2 % and CR rate was 34.6 %. Median time to response was 36 days (range, 13–217 days). Median time to response in breast/prostate cancer patients was 46 days (range, 13–217), whereas that in other cancer patients was 31 days (range, 14–73) (Fig. 1).

Analgesic use at 2 months after treatment was decreased for only 11.5 % of patients. With a median follow-up period of 6.8 months, median survival time was 6.1 months and the 1-year overall survival rate was 28.0 %. Median survival time was significantly longer in patients with breast or prostate cancer than in those with other malignancies (Fig. 2).

Next treatment after Sr-89

After Sr-89 treatment, 9 patients received chemotherapy as a next treatment. The remaining 43 patients received best supportive care. Of those, 12 patients received palliative radiotherapy for bone metastases; median time from Sr-89 to next radiotherapy was 48 days (range, 13–252 days).

Predictive factors

Age, primary site (breast/prostate vs. others), history of chemotherapy, and onset of flare-up response were

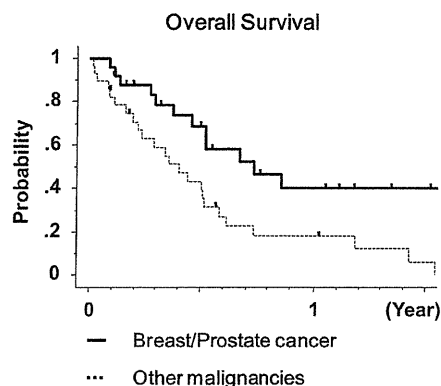


Fig. 2 Overall survival. Median survival time and 1-year survival rate were 8.0 months and 39.9 %, respectively, in patients with breast/prostate cancer, and 4.9 months and 17.9 % in those with other cancers. Overall survival was significantly longer in patients with breast/prostate cancer than in patients with other cancers ($p = 0.008$)

Table 3 Univariate analysis of predictive factors associated with response

	<i>n</i>	RR (%)	<i>P</i> value	HR (95 % CI)
Age (years)				1.01 (0.96–10.6)
Gender				
Male	23	73.9	0.70	0.78 (0.23–2.65)
Female	29	69		
Primary site				
Breast/prostate	26	72	0.76	1.21 (0.36–4.02)
Other	26	68.2		
Prior chemotherapy				
Yes	25	53.8	0.90	0.92 (0.28–3.07)
No	27	76.9		
Flare-up response				
Yes	13	53.8	0.12	2.86 (0.76–10.7)
No	39	76.9		

RR response rate, HR hazard ratio, CI confidence interval

investigated in univariate analysis (Table 3), but no significant predictive factor was identified.

Discussion

The clinical profile obtained in this study suggests that Sr-89 chloride may be of benefit in the treatment of painful bone metastases, not only in patients with breast and prostate cancer but also in those with various other malignancies.

Previous studies have identified hematological toxicity as one of the main side effects of Sr-89 chloride [6, 12, 13]. Hematological toxicities were present in the present study also but were of acceptable severity, albeit that the

frequency of anemia was slightly higher than in previous reports. Further, the incidence of flare-up response was higher than with radiotherapy. Previous reports have shown similar results [6, 12], which appears to support the hypothesis that the incidence of flare-up response increases with increasing volume of bone metastases.

Among other findings, overall response rate was 71.2 % and CR rate was 34.6 %. Overall response rate was 69.2 % in breast and prostate cancer and 73.1 % in other cancers. These results showed that Sr-89 chloride had definite benefit in patients with painful bone metastases.

Our present results are of particular clinical value given the relative paucity of information about the clinical profile of Sr-89 chloride in cancers other than breast and prostate.

In this study, we used self-reported outcome measures as response index, including pain diary data on a 0–10 numeric rating scale (NRS). However, analgesic use was decreased for only 11.5 % of all patients at 2 months after treatment, and considerable discrepancy was seen between the results calculated by the diary and interview response indexes.

In clinical practice, analgesic use is seldom decreased even when the patient reports a decrease in bone pain. There are two major reasons for this: first, analgesics may also be required for other pain; and second, a decrease in analgesic use carries the risk that pain will recur. The recurrence of pain is a fatal outcome, particularly in patients receiving best supportive care. For these reasons, because a change in the amount of analgesics used does not reflect the response to Sr-89 chloride, we consider that this variable should not be used as an index in clinical practice.

The predictive factors of response to Sr-89 are still controversial. Some investigators have found a better response in patients with good condition, and a poorer response with far-advanced metastatic disease [9, 14–16]. On the other hand, in some reports no significant difference was seen in patient background between responders and non-responders [17, 18].

In the present study, age, primary site (breast/prostate vs. other), history of chemotherapy, and onset of flare-up response were investigated in univariate analysis, but no significant predictive factor was identified.

The primary site of cancer and treatment history had no impact on the efficacy of Sr-89 chloride in univariate analysis, suggesting that Sr-89 chloride can be considered regardless of the primary site and treatment history.

Two major limitations of this study warrant mention. First, pain-free survival could not be evaluated because of the difficulty in conducting detailed and frequent interviews in patients receiving best supportive care. Second, the small scale and retrospective design of the study meant

that significant predictive factors of efficacy could not be adequately investigated.

Nevertheless, we consider that these results will be valuable for clinicians concerned with the difficult issue of bone pain control, particularly in view of the paucity of other data on this agent.

Conclusion

Sr-89 chloride may be useful in the treatment of bone metastasis pain in patients with various malignancies, as it is in those with breast and prostate cancer.

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Conflict of interest We have no conflict of interest.

Ethical standard In the present study, written informed consent for treatment was obtained from all patients before the initiation of treatment. This study was approved in Institutional Review Board of National Cancer Center Hospital, Japan.

References

1. Paes FM, Serafini AN (2010) Systemic metabolic radiopharmaceutical therapy in the treatment of metastatic bone pain. *Semin Nucl Med* 40:89–104
2. Taylor AJ Jr (1994) Strontium-89 for the palliation of bone pain due to metastatic disease. *J Nucl Med* 35:2054
3. Lam MG, de Klerk JM, van Rijk PP et al (2007) Bone seeking radiopharmaceuticals for palliation of pain in cancer patients with osseous metastases. *Anticancer Agents Med Chem* 7:381–397
4. Blake GM, Zivanovic MA, Blaquièrè RM et al (1988) Strontium-89 therapy: measurement of absorbed dose to skeletal metastases. *J Nucl Med* 29:549–557
5. Fuster D, Herranz D, Vidal-Sicart S et al (2000) Usefulness of strontium-89 for bone pain palliation in metastatic breast cancer patients. *Nucl Med Commun* 21:623–626
6. Turner SL, Gruenewald S, Spry N et al (2001) Less pain does equal better quality of life following strontium-89 therapy for metastatic prostate cancer. *Br J Cancer* 84:297–302
7. Baczyk M, Milecki P, Baczyk E et al (2003) The effectiveness of strontium-89 in palliative therapy of painful prostate cancer bone metastases. *Ortop Traumatol Rehabil* 5:364–368
8. Ashayeri E, Omogbehin A, Sridhar R et al (2002) Strontium-89 in the treatment of pain due to diffuse osseous metastases: a university hospital experience. *J Natl Med Assoc* 94:706–711
9. Kraeber-Bodere F, Campion L, Rousseau C et al (2000) Treatment of bone metastases of prostate cancer with strontium-89 chloride: efficacy in relation to the degree of bone involvement. *Eur J Nucl Med* 27:1487–1493
10. Paice JA, Cohen FL (1997) Validity of a verbally administered numeric rating scale to measure cancer pain intensity. *Cancer Nurs* 20:88–93
11. Williamson A, Hoggart B (2005) Pain: a review of three commonly used pain rating scales. *J Clin Nurs* 14:798–804
12. Gunawardana DH, Lichtenstein M, Better N et al (2004) Results of strontium-89 therapy in patients with prostate cancer resistant to chemotherapy. *Clin Nucl Med* 29:81–85
13. Smeland S, Erikstein B, Aas M et al (2003) Role of strontium-89 as adjuvant to palliative external beam radiotherapy is questionable: results of a double-blind randomized study. *Int J Radiat Oncol Biol Phys* 56:1397–1404
14. Windsor PM (2001) Predictors of response to strontium-89 (Metastron) in skeletal metastases from prostate cancer: report of a single centre's 10-year experience. *Clin Oncol (R Coll Radiol)* 13:219–227
15. Laing AH, Ackery DM, Bayly RJ et al (1991) Strontium-89 chloride for pain palliation in prostatic skeletal malignancy. *Br J Radiol* 64:816–822
16. Schmeler K, Bastin K (1996) Strontium-89 for symptomatic metastatic prostate cancer to bone: recommendations for hospice patients. *Hosp J* 11:1–10
17. Firusian N (1978) Endo-osseous isotope therapy of malignant skeletal disease (author's translation). *Z Krebsforsch Klin Onkol Cancer Res Clin Oncol* 91:143–156
18. Silberstein EB, Williams C (1985) Strontium-89 therapy for the pain of osseous metastases. *J Nucl Med* 26:345–348

RET-targeting molecular stratified non-small-cell lung cancers

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Abstract: Recent advances in lung cancer genomics have successfully characterized therapeutic targets of lung cancer. *RET* fusion gene products are among the newest target molecules for lung adenocarcinoma. Preclinical findings and preliminary reports regarding potential tumor control by RET-targeting multi-kinase inhibitors encourage further clinical trials. The infrequent prevalence of *RET* fusion gene-positive cases may be a major obstacle hindering the development of RET-targeted therapy. Thus, it is necessary to recruit appropriate participants for trials to develop an efficient *RET* fusion gene detection system to achieve targeted therapy for lung adenocarcinomas stratified by this molecular target.

Keywords: Lung adenocarcinoma; *RET* fusion gene; clinical trial



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Cancer genomics studies involving next generation sequencing (NGS) technology have successfully characterized therapeutic targets of lung cancer. Among lung adenocarcinoma genomes, activating mutations in *EGFR*, *ERBB2*, *KRAS* and *BRAF* as well as gene fusions of *ALK*, the products of which activate the autonomous proliferation of cancer cells via the Ras-MAPK pathway, have been regarded as so-called “driver mutations” (1). It is also known that these alterations exist in a mutually exclusive manner. In addition to these well-characterized driver mutations, independent groups from Japan, Korea and the USA recently found novel chromosome ten inversions that produce fusion genes containing the receptor tyrosine kinase encoding gene *RET* (2-5). Three of four groups applied NGS to determine the fusion genes. Kohno *et al.* and Ju *et al.* used cDNA samples from known driver-negative lung adenocarcinoma specimens for whole transcriptome sequencing to screen novel fusion gene products (2,3). Lipson *et al.* designed a custom target capture genomic DNA sequencing panel targeting the exons and introns of genes encoding previously reported cancer-related kinases and screened lung and colorectal cancer genomes (5).

By using different strategies, these groups identified the

same *KIF5B-RET* fusion gene. In these primary reports, the authors found that *RET* fusion gene products are aberrantly expressed in tumor cells. Exogenously overexpressed *RET* fusion kinases are constitutively active and have transforming activity. Multi-kinase inhibitors, which reportedly inhibit *RET*, effectively suppress the growth advantage and transforming activity of *RET* fusion kinases (3-5). Further screening of lung adenocarcinoma-derived cell lines found that LC/2-ad cells, which were established from a pleural effusion from a Japanese patient with lung adenocarcinoma, expressed a *CCDC6-RET* fusion gene (6,7). In addition to experiments with exogenously overexpressed fusion *RET*, vandetanib, a *RET*-inhibiting multi-kinase inhibitor, successfully inhibited downstream signals and exhibited significant anti-tumor effects *in vitro* and *in vivo*.

These findings strongly encourage the development of *RET*-targeted therapy for lung adenocarcinoma. Currently, five independent, open-label, single-arm, phase II studies have begun to assess the therapeutic effects of vandetanib (ZD6474), cabozantinib (XL184), lenvatinib (E7080) and ponatinib (AP24534) (Table 1). Drilon *et al.* reported promising results for the first three cases of their clinical trial, which investigated the efficacy of cabozantinib (8). In addition, a case report from Switzerland reported that

Table 1 Ongoing clinical trials of RET-targeting therapies with RET fusion gene-positive NSCLC

Trial ID	Drug (pharmaceutical company)	Study design	Primary end-point	Enrolment (cases)	Study start
NCT01639508	Cabozantinib/XL184 (Exelixis)	Open-label, single arm	Response rate	25	July 2012
UMIN000010095	Vandetanib/ZD6474 (AstraZeneca)	Open-label, single arm	Response rate	17	February 2013
NCT01823068	Vandetanib/ZD6474 (AstraZeneca)	Open-label, single arm	Response rate	17	April 2013
NCT01877083	Lenvatinib/E7080 (Eisai)	Open-label, single arm	Response rate	20	April 2013
NCT01813734	Ponatinib/AP24534 (ARIAD)	Open-label, single arm	Response rate	20	June 2013

vandetanib induced the remission of metastatic *KIF5B-RET* fusion gene-positive lung tumors (9). However, the infrequent prevalence of *RET* fusion gene-positive cases is a major obstacle hindering the further development of RET-targeted therapy. Primary and subsequent studies including a report by Wang *et al.* screened approximately 5,000 lung adenocarcinoma cases in total (10,11). *RET* fusion gene-positive cases were found in 1-2% of all non-small cell lung cancer (NSCLC) patients in Asian and European populations. Based on these estimations, more than 1,000 cases must be screened to identify 10 to 20 *RET* fusion gene-positive cases for proof-of-concept phase II studies. When efficacy is estimated with studies involving a larger number of cases, the number of pre-screening participants is also greater.

The clinicopathological features that characterize *RET* fusion gene-positive cases may help identify patients who should be subjected to further genetic screening. Most of the positive cases are adenocarcinomas, but several cases involve other histological types of NSCLC, such as adenosquamous carcinoma. The *RET* fusion is most likely to occur in young and/or never/light-smoker patients. Lung adenocarcinomas harboring *KIF5B-RET* fusions have well or moderately differentiated histological features similar to those harboring *EGFR* mutations, whereas lung adenocarcinomas harboring *CCDC6-RET* fusions often have signet-ring and mucinous cribriform features similar to *EML4-ALK* fusion-positive lung adenocarcinomas (10,11). These findings suggest a difficulty in distinguishing *RET* fusion gene-positive lung adenocarcinomas from commonly observed lung adenocarcinomas in Asian countries by histopathological diagnosis. Thus, appropriate genetic testing is mandatory for selecting *RET* fusion gene-positive cases.

Investigators have made much effort progress in recruiting adequate numbers of participants for prescreening for the above-mentioned phase II studies. The LURET (Lung Cancer with RET rearrangement) study, led by Dr. Koichi Goto at National Cancer Center Japan

(UMIN00001009), evaluates the efficacy of vandetanib in 17 patients with *RET* fusion gene-positive NSCLC. The multi-kinase inhibiting spectrum of vandetanib includes *EGFR*, and *VEGFR* and *RET*. Although the therapeutic efficacy of vandetanib in advanced NSCLC patients was previously evaluated in "all-comer" clinical trials, significantly better therapeutic effects of vandetanib compared to pre-existing therapeutic regimens was not shown. We assume that another clinical trial recruiting only *RET* fusion gene-positive cases is necessary to evaluate the vandetanib effects. To recruit participants, a consortium designated LC-SCRUM (Lung Cancer Genomic Screening Project for Individualized Medicine in Japan) has been established. In LC-SCRUM, frozen biopsy tissues or pleural effusions from patients with non-squamous NSCLC without an *EGFR* mutation are curated from 136 hospitals throughout Japan, and *RET* fusion genes are detected using a combination of RT-PCR and FISH. Multiplex RT-PCR primers are designed to detect all of the previously described *KIF5B-RET* and *CCDC6-RET* variants. The positive cases are then subjected to break-apart and fusion FISH to validate the RT-PCR results. Cases positive by RT-PCR and FISH are eligible for the LURET study.

As Wang *et al.*, mentioned in their report, standard methods for the detection of gene fusions, including RT-PCR, FISH and immunohistochemistry (IHC), have difficulty detecting *RET* fusion genes and their products (10). RT-PCR exhibits preferable sensitivity and specificity for detecting known fusion gene cDNA, but it is usually insufficient for detecting new partners or isoforms. Anti-*RET* antibodies that specifically distinguish overexpressed *RET* fusion proteins have not been generated. Although FISH is currently the most effective diagnostic technology for detecting chromosomal rearrangements, the high cost and need for technical expertise limit its practical application.

We should also take into account the cost efficiency of *RET* fusion gene detection, which only benefits the 1% of

cases with a *RET* fusion gene. The detection of rare fusion genes is not just a pecuniary loss, it also wastes precious tissue samples obtained by biopsy or archived surgically resected specimens. To resolve these difficulties, genomic testing of lung adenocarcinoma driver mutations should evolve from single gene testing to multiplex genetic testing. Several technologies, including digital PCR and NGS-based target-capture sequencing, should be preferable candidates for future *in vitro* diagnostic systems. Although these technologies are still immature in their robustness and cost efficiency, these next-generation technologies must be positively applied to clinical diagnosis and may help in establishing a basis for the development of targeted therapy for lung cancer treatment.

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References

- Mitsudomi T, Suda K, Yatabe Y. Surgery for NSCLC in the era of personalized medicine. *Nat Rev Clin Oncol* 2013;10:235-44.
- Ju YS, Lee WC, Shin JY, et al. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. *Genome Res* 2012;22:436-45.
- Kohno T, Ichikawa H, Totoki Y, et al. KIF5B-RET fusions in lung adenocarcinoma. *Nat Med* 2012;18:375-7.
- Takeuchi K, Soda M, Togashi Y, et al. RET, ROS1 and ALK fusions in lung cancer. *Nat Med* 2012;18:378-81.
- Lipson D, Capelletti M, Yelensky R, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. *Nat Med* 2012;18:382-4.
- Matsubara D, Kanai Y, Ishikawa S, et al. Identification of CCDC6-RET fusion in the human lung adenocarcinoma cell line, LC-2/ad. *J Thorac Oncol* 2012;7:1872-6.
- Suzuki M, Makinoshima H, Matsumoto S, et al. Identification of a lung adenocarcinoma cell line with CCDC6-RET fusion gene and the effect of RET inhibitors *in vitro* and *in vivo*. *Cancer Sci* 2013;104:896-903.
- Drilon A, Wang L, Hasanovic A, et al. Response to Cabozantinib in patients with RET fusion-positive lung adenocarcinomas. *Cancer Discov* 2013;3:630-5.
- Gautschi O, Zander T, Keller FA, et al. A patient with lung adenocarcinoma and RET fusion treated with vandetanib. *J Thorac Oncol* 2013;8:e43-4.
- Wang R, Hu H, Pan Y, et al. RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. *J Clin Oncol* 2012;30:4352-9.
- Kohno T, Tsuta K, Tsuchihara K, et al. RET fusion gene: Translation to personalized lung cancer therapy. *Cancer Sci* 2013. [Epub ahead of print].

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Effect of a poly(ADP-ribose) polymerase-1 inhibitor against esophageal squamous cell carcinoma cell lines

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Key words

DNA repair, esophageal cancer, poly(ADP-ribose) polymerase inhibitor, RNF8, γ -H2AX

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Effective molecular target drugs that improve therapeutic efficacy with fewer adverse effects for esophageal cancer are highly anticipated. Poly(ADP-ribose) polymerase (PARP) inhibitors have been proposed as low-toxicity agents to treat double strand break (DSB)-repair defective tumors. Several findings imply the potential relevance of DSB repair defects in the tumorigenesis of esophageal squamous cell carcinoma (ESCC). We evaluated the effect of a PARP Inhibitor (AZD2281) on the TE-series ESCC cell lines. Of these eight cell lines, the clonogenic survival of one (TE-6) was reduced by AZD2281 to the level of DSB repair-defective Capan-1 and HCC1937 cells. AZD2281-induced DNA damage was implied by increases in γ -H2AX and cell cycle arrest at G2/M phase. The impairment of DSB repair in TE-6 cells was suggested by a sustained increase in γ -H2AX levels and the tail moment calculated from a neutral comet assay after X-ray irradiation. Because the formation of nuclear DSB repair protein foci was impaired in TE-6 cells, whole-exome sequencing of these cells was performed to explore the gene mutations that might be responsible. A novel mutation in RNF8, an E3 ligase targeting γ -H2AX was identified. Consistent with this, polyubiquitination of γ -H2AX after irradiation was impaired in TE-6 cells. Thus, AZD2281 induced growth retardation of the DSB repair-impaired TE-6 cells. Interestingly, a strong correlation between basal expression levels of γ -H2AX and sensitivity to AZD2281 was observed in the TE-series cells ($R^2 = 0.5345$). Because the assessment of basal DSB status could serve as a biomarker for selecting PARP inhibitor-tractable tumors, further investigation is warranted.

Esophageal carcinoma is the sixth most common cause of cancer-related deaths worldwide and is associated with a poor prognosis.⁽¹⁾ Surgical therapies of resectable esophageal cancer exhibit a 5-year survival rate ranging from 20% to 27%.^(2–4) Less invasive therapies that preserve the esophagus have also been introduced. Endoscopic therapies, such as endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), have been adopted for early esophageal cancer and have achieved favorable outcomes, but postoperative esophageal stricture frequently occurs after these treatments. Furthermore, intensive follow-up is necessary to manage new heterochronous lesions.^(5–7) Chemoradiotherapy (CRT), which combines radiation, 5-fluorouracil (5-FU) and cisplatin (CDDP), is a promising therapeutic alternative to esophagectomy with a survival rate equivalent to that of surgical therapies.^(8,9) However, the acute and late adverse effects of chemoradiotherapy, including pancytopenia and pneumonitis, still require consideration. There is a demand for effective molecular target drugs for esophageal cancer that combine an improved therapeutic efficacy with fewer adverse effects.

Poly(ADP-ribose) polymerase (PARP) inhibitors induce the accumulation of DNA single-strand breaks (SSB), which

cause the formation of DNA double-strand breaks (DSB) after the stalling and collapse of progressing DNA replication forks.⁽¹⁰⁾ Though DSBs are repaired by the error-free homologous recombination repair (HRR) pathway in non-tumor cells, they remain unrepaired and induce lethality in HRR-defective tumor cells.^(11,12) Based on this mechanism, PARP inhibitors have been proposed as low toxicity agents for HRR-defective tumors. BRCA1 and BRCA2 are key components of the HRR machinery, and the abnormality of these genes is known to cause sporadic and hereditary breast and ovarian cancers.⁽¹³⁾ Consistent with this, PARP inhibitors have been developed for breast and ovarian cancers. In addition, an increasing number of biomarker candidates that predict the sensitivity of a tumor to PARP inhibitors have been reported.^(14–18)

Esophageal carcinoma is histologically classified into squamous cell carcinoma (ESCC) and adenocarcinoma; the former is common in East Asia. Although the direct relevance has not been well investigated, several findings suggest that a defect in the HRR pathway contributes to the tumorigenesis of ESCC. The risk of esophageal and head and neck squamous carcinoma is increased among Fanconi anemia (FA)

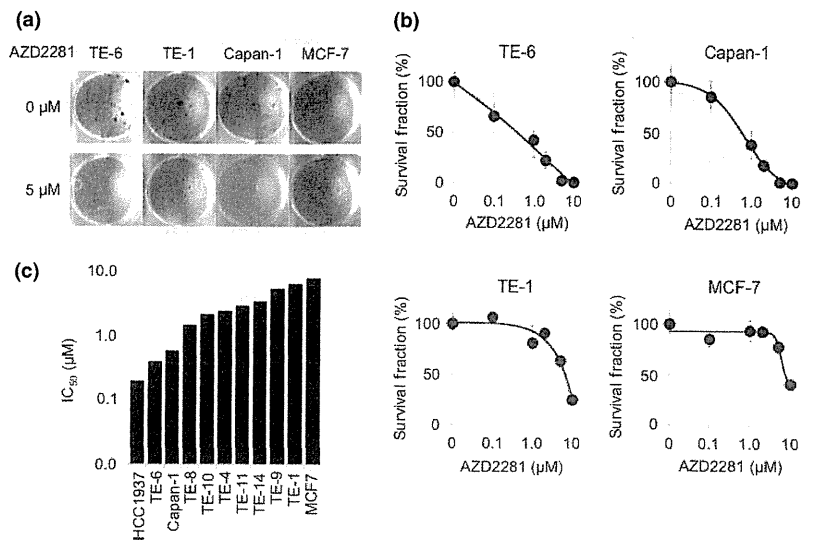


Fig. 1. Sensitivity of TE-series cell lines to a poly (ADP-ribose) polymerase (PARP) inhibitor, AZD2281. (a) TE-1, TE-6, Capan-1 (BRCA2-deficient) and MCF7 (wild-type BRCA) cells were treated with or without AZD2281 at the indicated concentrations for at least seven doubling times. Cells were fixed and stained with crystal violet and the number of colonies was counted. (b) Sensitivity to AZD2281 was evaluated by clonogenic assay. Colonies consisting of more than 64 cells were counted and the survival fraction was estimated. Three independent experiments were carried out. The data represent the average and standard deviations. (c) IC₅₀ (μM) value of AZD2281 in eight TE series, MCF7, HCC1937 and Capan-1 cells measured by the clonogenic assays.

patients whose HRR pathway was disturbed due to FA-predisposing gene alterations.^(19,20) In addition, recently reported whole-exome sequencing data from 74 head and neck SCCs revealed that more than half of SCC cases harbored mutations in genes involved in DNA repair.⁽²¹⁾ Therefore, we assume that some fraction of ESCCs harbor DSB repair defects and might be favorable targets of PARP inhibitors. The aim of this study was to examine the efficacy of a potent PARP-1 inhibitor in a series of ESCC cell lines established from Japanese patients.

Materials and Methods

Complete materials and methods were described in the supplementary information (Data S1).

Purchased materials. A PARP inhibitor, AZD2281 (Olaparib) and BSI-201 (Iniparib) were purchased from Selleck Chemicals (Houston, TX, USA). The TE-1, TE-4, TE-6, TE-8, TE-9, TE-10, TE-11 and TE-14 cell lines were purchased from the Riken BioResource Center (Tsukuba, Japan). The Capan-1, HCC1937, MDA-MB-436 and MCF-7 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Clonogenic assays. A total of 500–2000 cells were cultured with AZD2281- or vehicle-containing media. After 10–16 days, cells were fixed and stained with crystal violet. Colonies consisting of more than 64 cells were subsequently counted.

Immunoblotting analysis. The treated cell lysates were separated by 15% SDS-PAGE and the blot was hybridized with the

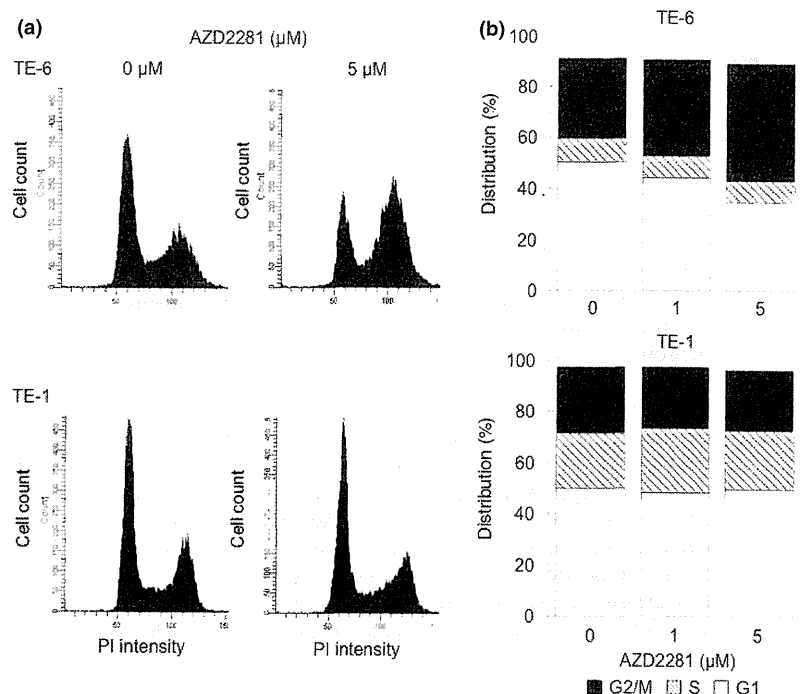


Fig. 2. AZD2281-induced G2/M arrest in TE-6 cells. (a) TE-6 and TE-1 cells were cultured with or without AZD2281 for 24 h. DNA ploidy was assessed by propidium iodide (PI) staining and flow cytometry. (b) The proportion of estimated cell-cycle phases in TE-6 and TE-1 cells treated with or without AZD2281. The data represent the average of three independent experiments.

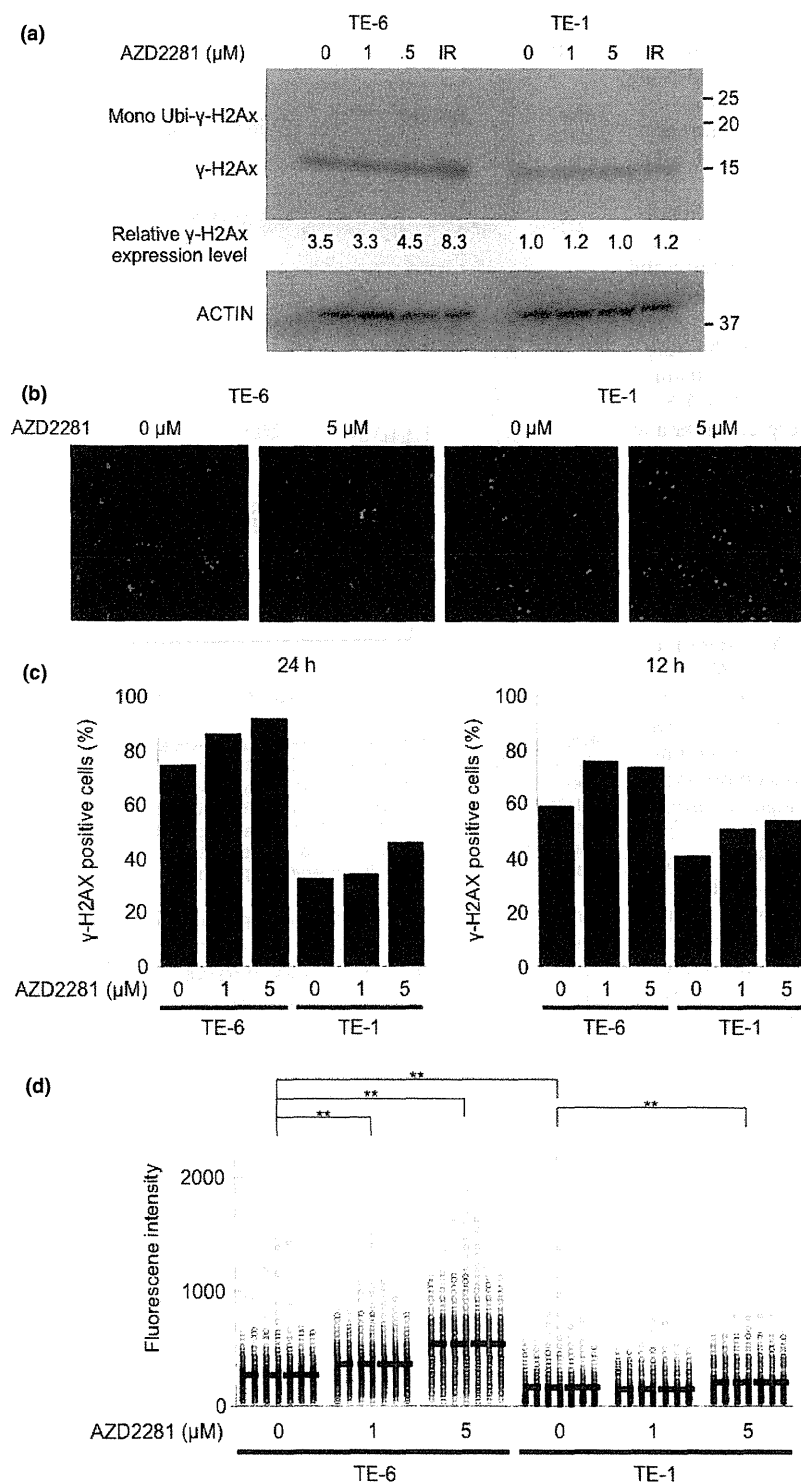


Fig. 3. Increase in double strand breaks (DSBs) in TE-6 cells treated with AZD2281. (a) TE-6 and TE-1 cells were treated with AZD2281 for 24 h and with 5 Gy X-ray irradiation, and γ-H2AX was assessed using Western blotting. The anti-γ-H2AX antibody detected both unubiquitinated (15 kD) and mono-ubiquitinated (23.6 kD) γ-H2AX. (b) TE-6 and TE-1 cells were treated with AZD2281 for 24 h and γ-H2AX was assessed by immunofluorescence. DAPI (blue) and γ-H2AX (red) images were superimposed. (c) Number of the γ-H2AX-positive TE-6 and TE-1 cells treated with or without AZD2281 at the indicated concentrations for 24 h. (d) Scatter diagrams show the fluorescence intensity of individual TE-6 and TE-1 cells treated with or without AZD2281 at the indicated concentrations for 24 h. The lines shown indicated the averages of the data plotted. The data were obtained from at least 500 cells for each condition. ***P* < 0.01 (Student's *t*-test).

phospho-Histone H2A.X (Ser139) (20E3) rabbit monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) and then with a HRP-conjugated secondary antibody (1:50000; Santa Cruz Biotechnology, Dallas, TX, USA). Densitometric analysis was performed with Image-J software (<http://imagej.nih.gov/ij/>).

Immunofluorescence analysis. To evaluate the formation of DSBs, cells grown on 96-well plates were treated with an

anti-γ-H2AX rabbit monoclonal antibody (Cell Signaling Technology) followed by a goat anti-Mouse IgG (H + L) DyLight 549-conjugated secondary antibody (Thermo Scientific, Waltham, MA, USA). γ-H2AX was observed under an ArrayScan HCS System (Thermo Scientific). To evaluate the formation of 53BP1 and RAD51 nuclear foci, cells grown on μ-Dish³⁵ mm, low (ibidi) were treated with an anti-53BP1 rabbit polyclonal antibody (Abcam, Cambridge, UK) or an anti-RAD51 rabbit

polyclonal antibody (Santa Cruz Biotechnology) followed by an Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (Invitrogen, Carlsbad, CA, USA). These foci were observed under a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan).

Neutral comet assays. Neutral comet assays were performed using the CometAssay kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Cell cycle analysis. The cells were fixed with 70% ethanol and stained with propidium iodide. The DNA content of the cells was evaluated using a FACS CantoII flow cytometer and FACS Diva software (BD, Franklin Lakes, NJ, USA).

Whole-exome sequencing. Targeted enrichment was performed using the SureSelect Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced using Illumina Genome Analyzer IIx (Illumina, San Diego, CA, USA).

Statistical analysis. Individual experiments were performed at least in triplicate. The statistical significance of observed differences was analyzed using Student's *t*-test. One asterisk (*) indicates a *P*-value smaller than 0.05. Two asterisks (**) indicate a *P*-value smaller than 0.01.

Results

Sensitivity of TE series cell lines to AZD2281. We tested the sensitivity of the TE series of ESCC cell lines to AZD2281 using clonogenic assays. All of the cell lines were cultured with various concentrations of AZD2281 for at least seven doubling times (10–16 days), and the number of colonies with more than 64 cells was counted. The concentrations of AZD2281 that caused a 50% reduction in clonogenic survival (IC_{50}) in the TE-6, TE-8, TE-10, TE-4, TE-11, TE-14, TE-9, and TE-1 cells were 0.4, 1.5, 2.2, 2.5, 3.0, 3.5, 5.5 and 6.5 μ M, respectively (Figs 1a–c, S1). The IC_{50} values for HCC1937 and Capan-1 cells, which have deletions or mutations in the BRCA genes and are reported to be sensitive to PARP inhibitors,^(22,23) were 0.2 and 0.6 μ M, respectively. The IC_{50} values for MCF7 cells, which have wild-type BRCA genes and are known to be resistant to PARP inhibitors, was 8.0 μ M. We further tested another PARP inhibitor, BSI-201. The IC_{50} values for TE-6 and TE-1 cells were 9.6 and 22.0 μ M, respectively (Fig. S2). Because AZD2281 and BSI-201 suppressed the growth of the TE-6 cells as efficiently as it suppressed the growth of the BRCA-deficient, PARP-inhibitor-sensitive cell lines and failed to suppress the growth of the TE-1 cells; we designated TE-6 as a PARP inhibitor-sensitive ESCC cell line and TE-1 as a PARP inhibitor-resistant cell line. We selected these cells for further analyses.

AZD2281-induced G2/M arrest in TE-6 cells. To further study the mechanism of growth retardation of TE-6 cells by AZD2281, the status of the cell cycle in these cells was assessed by analyzing the DNA ploidy pattern (Figs 2a,b, S3). Treatment with 1 and 5 μ M of AZD2281 for 12 h increased the population with 4n DNA content from 36.7% to 40.7% and 40.6%, respectively. Treatment for 24 h further increased the population with 4n DNA content from 31.6% to 37.9% and 46.3%, respectively. This suggested an increase in the G2/M or M arrested population after AZD2281 treatment. On the other hand, no significant increase of tetraploid cells was observed in the TE-1 cells.

Increase of DSBs in TE-6 cells treated with AZD2281. To determine whether DSBs are formed after treatment with AZD2281 for 24 h, we assessed the amount of γ -H2AX as a marker of DSBs. Western blotting revealed that the level of γ -H2AX

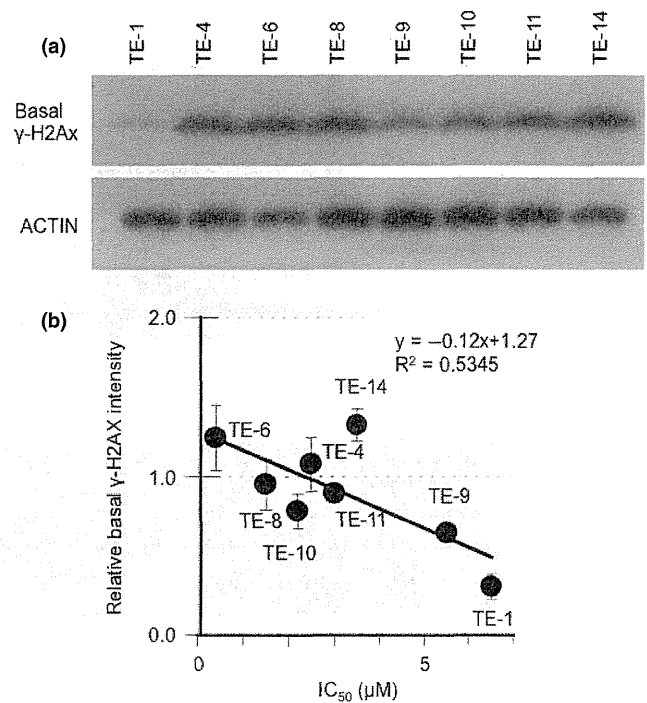


Fig. 4. A strong correlation between base-level γ -H2AX and sensitivity to AZD2281 of TE cells. (a) Eight non-treated TE-series cell lines were subjected to Western blot analysis with antibodies against γ -H2AX and actin. (b) The correlation between basal γ -H2AX expression levels and IC_{50} of AZD2281. The average intensity of γ -H2AX was standardized with actin. The data represent the averages and standard deviations of three independent experiments.

staining in TE-6 cells increased in a dose-dependent manner. However, no such increase was observed in TE-1 cells (Fig. 3a). The same trend was observed by immunofluorescence. Both the percentage of γ -H2AX positive cells determined by visual inspection and the average fluorescence intensity of γ -H2AX staining per cell increased significantly in a dose-dependent manner in TE-6 cells, but not in TE-1 cells, suggesting that AZD2281 induced an accumulation of DNA damage in TE-6 cells (Fig. 3b–d).

A strong correlation between base-level γ -H2AX and sensitivity to AZD2281 of TE cells. The Western blotting and immunofluorescence data also suggested that the baseline γ -H2AX level was higher in the AZD2281-sensitive TE-6 cells than in the resistant TE-1 cells (Fig. 3a). Because the increased amount of γ -H2AX may suggest the accumulation of DSBs, we evaluated the correlation between the basal expression levels of γ -H2AX and sensitivity to AZD2281 among the eight TE cell lines. A significant correlation between the basal levels of γ -H2AX and the IC_{50} of AZD2281 was observed ($R^2 = 0.5345$) (Fig. 4a,b).

Sustained X-ray irradiation-induced DSBs in TE-6 cells. To assess whether the impairment of DSB repair is relevant to the sensitivity of TE-series cells to AZD2281, we evaluated X-ray

Table 1. Tail moment of the X-ray-irradiated TE-6 and TE-1 cells

	Time after irradiation			
	0 h	15 min	2 h	6 h
TE-6	2.43 \pm 3.19	5.27 \pm 4.35	9.78 \pm 6.27	13.7 \pm 8.20
TE-1	3.69 \pm 4.14	5.56 \pm 3.97	5.20 \pm 4.22	3.20 \pm 4.71

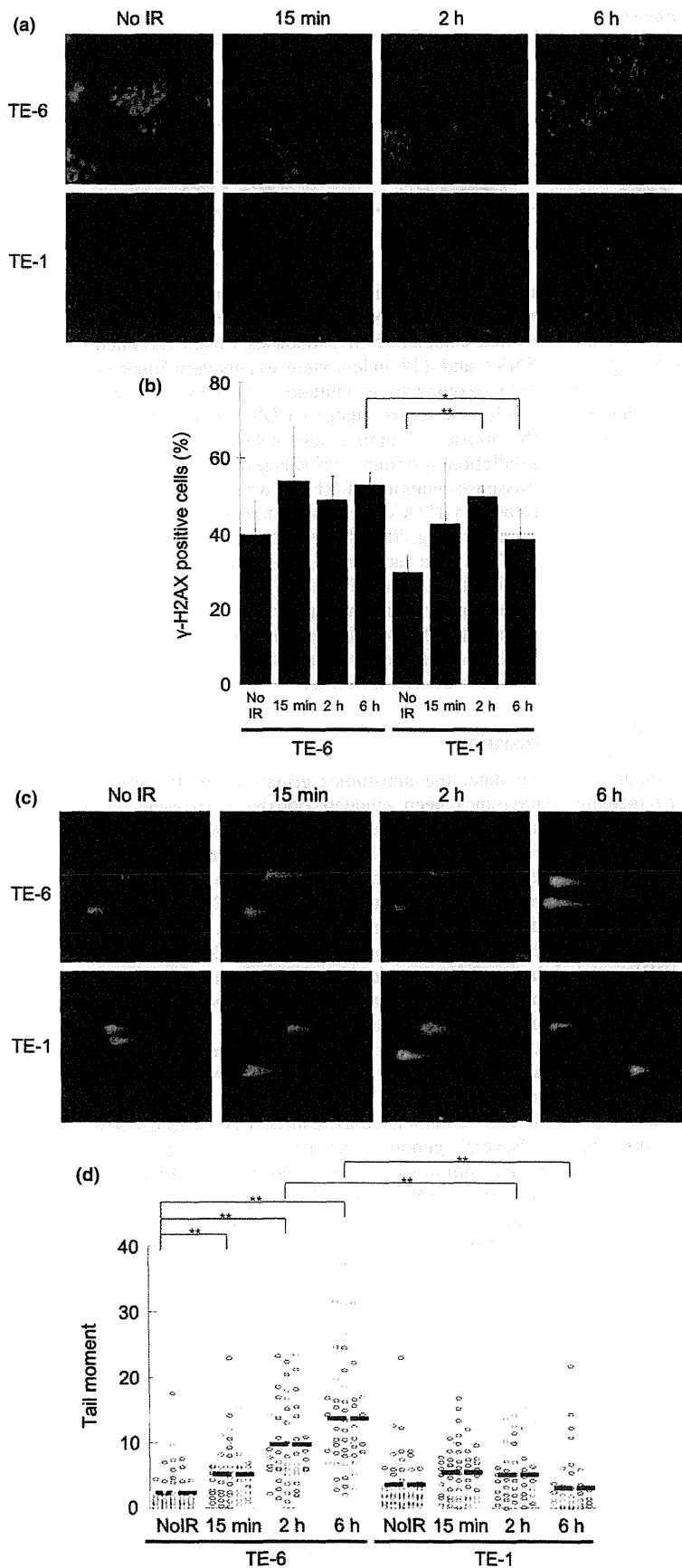


Fig. 5. Sustained X-ray irradiation-induced double strand breaks (DSBs) in TE-6 cells. (a) TE-6 and TE-1 cells were irradiated with 2 Gy X-ray. Cells were stained with anti- γ -H2AX antibody 15 min, 2 and 6 h after irradiation. (b) Percentages of γ -H2AX-positive cells. The data represent the average and standard deviations of three independent experiments. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). (c) Representative neutral comet assay results of TE-6 and TE-1 cells treated with or without 5 Gy of X-ray irradiation. (d) Scatter diagrams show the tail moment of individual TE-6 and TE-1 cells treated with or without 5 Gy of X-ray irradiation. The lines shown indicate the averages of the data plotted. The data were obtained from at least 100 cells for each condition. * $P < 0.01$ (Student's *t*-test).

Table 2. The number of nuclear foci of the DNA repair proteins

X-Ray (Gy)	53BP1			Rad51	
	0	2		0	10
Time	–	15 min	2 h	–	2 h
TE-6	0.55 ± 1.44	0.94 ± 1.12	1.48 ± 1.20	0.67 ± 2.20	2.25 ± 3.20
TE-1	1.09 ± 1.58	2.48 ± 2.44	4.86 ± 3.23	1.11 ± 1.50	3.65 ± 3.59

irradiation-induced DNA DSBs in these cells. First, the amount of γ -H2AX was assessed by immunofluorescence. As mentioned above, the γ -H2AX level of TE-6 cells was high at baseline and increased 15 min after irradiation and sustained for 6 h. In TE-1 cells, increase of γ -H2AX level was observed 2 h after irradiation, but declined after 6 h (Fig. 5a,b). Next, a neutral comet assay was performed to assess DNA damage. This assay detects a wide range of DNA lesions, including DSBs; the tail moment parameter is used as an index of DNA damage. We found a sustained increase in the tail moment of TE-6 cells (irradiation [–]; 2.43 ± 3.19, 15 min; 5.27 ± 4.35 2 h; 9.78 ± 6.27, 6 h; 13.71 ± 8.20), while in TE-1 cells, the tail moment transiently increased at 2 h after 5 Gy X-ray irradiation and then returned to the basal level at 6 h (irradiation [–]; 3.69 ± 4.14, 15 min; 5.56 ± 3.97 2 h; 5.20 ± 4.22, 6 h; 3.20 ± 4.71) (Fig. 5c,d, Table 1). These results suggested that the X-ray irradiation-induced DNA damage was properly repaired in the TE-1 cells but was sustained in the TE-6 cells.

Impairment of DSB repair protein nuclear foci formation in X-ray irradiated TE-6 cells. Because the TE-6 cells were found to be defective in DSB repair, we assessed the amount of BRCA1/2 expression in TE-series cells by Western blotting (Fig. S4a,b). However, no significant difference in the expression of BRCA1/2 in TE-6 and TE-1 cells was observed. To confirm the impairment of DNA repair machinery of TE-6 cells, we evaluated the nuclear focus formation of 53BP1, which is recruited to the γ -H2AX sites at an early stage in DSB repair, and RAD51, which is recruited at a late stage in DSB repair.^(24–26) The baseline expression levels of 53BP1 and RAD51 were higher in the TE-6 cells (Fig. S5). However, increase of the number of 53BP1 nuclear foci per cell was much less in the TE-6 cells (irradiation [–]; 0.55 ± 1.44, 15 min; 0.94 ± 1.12 2 h; 1.48 ± 1.20), whereas 53BP1 foci were increased in the TE-1 cells (irradiation [–]; 1.09 ± 1.58, 15 min; 2.48 ± 2.44 2 h; 4.86 ± 3.23) (Fig. 6a,b, Table 2). Similarly, 6 h after 10 Gy X-ray irradiation, the number of RAD51 foci per cell was significantly increased in the TE-1 cells (irradiation [–]; 1.11 ± 1.50, 6 h; 3.65 ± 3.59), whereas the increase in RAD51 foci was not significant in the TE-6 cells (irradiation [–]; 0.67 ± 2.20, 2 h; 2.25 ± 3.20) (Fig. 6c,d, Table 2). These results suggested that the interaction between γ -H2AX and 53BP1 and the subsequent recruitment of RAD51 were impaired in TE-6 cells.

A novel mutation in the RNF8 gene, and the reduced ability of TE-6 cells to polyubiquitinate γ -H2AX. To identify the molecular mechanism underlying the impaired DNA repair in TE-6 cells, we performed whole-exome sequencing of TE-6 and TE-1 cells and selected the DNA repair-related genes that were mutated in the genomic DNA of TE-6 cells but not TE-1 cells. In total, 16 722 and 16 543 single nucleotide variants (SNV) were identified from the exomes of TE-1 and TE-6 cells, respectively (Tables S1 and S2). Another 260 and 240 indels were identified from TE-1 and TE-6 cells, respectively. To

reduce the probable germline variants, the single nucleotide polymorphisms (SNPs) that were registered in the dbSNP and in-house Japanese SNP databases were eliminated. Finally, 606 SNVs and 118 indels were exclusively identified in the TE-6 cells. Among these mutations were hits in six genes (listed in Table 3) that are related to DNA repair. We further evaluated the impact of amino acid substitutions using the Polyphen2 prediction program; we focused in particular on the T448M missense mutation of RNF8, which is an E3-ligase polyubiquitinating γ -H2AX. The T448M mutation was close to the RING domain (Fig. 7a). To assess the ubiquitination status of γ -H2AX, we examined X-ray-irradiated TE-6 and TE-1 cells by Western blotting (Fig. 7b). In the TE-1 cells, the levels of mono- and di-ubiquitinated γ -H2AX were increased at 2 h after irradiation and decreased at 6 h after irradiation. Meanwhile, no significant increase in ubiquitination was observed in the TE-6 cells (Fig. 7b–d).

Discussion

To date, the anti-tumor effects of PARP inhibitors to ESCC have not been evaluated both *in vitro* and *in vivo*. In this study, we identified TE-6 cells as AZD2281-sensitive cell line for the first time. The following findings support the idea that the PARP inhibitor induced growth retardation in the DSB repair-impaired background of TE-6 cells: (i) AZD2281 induced the accumulation of DNA damage, as evaluated by γ -H2AX expression levels. (ii) AZD2281 induced the accumulation of a tetraploid cell population in a time-dependent manner, consistent with previous reports that cells with accumulated unrepaired DSBs caused in every S-phase are arrested at G2/M boundary.⁽²⁷⁾ (iii) Sustained damage to DNA, the attenuated ubiquitination of γ -H2AX and a defect in DSB repair nuclear foci formation under X-ray irradiation suggest that TE-6 cells have an impaired DNA repair ability.

Several genomic biomarkers that predict the efficacy of PARP inhibitors have been identified. Though the presence or absence of *BRCA1* and *BRCA2* alterations most strikingly distinguishes the PARP inhibitor-sensitive cells and patients, no pathogenic mutations in either gene were identified in the TE-6 cells (Table S1). Consistent with this, expression of *BRCA1* and *BRCA2* proteins were not reduced in TE-6 cells (Fig. S3a,b). The alteration of other genes, such as *53BP1*, *RAD51*, *PTEN* and *USP11*, also reportedly affects the sensitivity to PARP inhibitors.^(18,28–32) To identify the genomic events corresponding to the AZD2281 sensitivity of TE-6 cells, we performed whole-exome sequencing. Because a paired non-tumor genome is not available for the TE-series cells, we subtracted previously identified germline variants from our set of total SNVs and indels to enrich for potential somatic mutations. Among the remaining mutations, we focused on those located in the genes encoding DNA repair-related proteins and identified a novel mutation of *RNF8*. *RNF8* is an E3-ligase

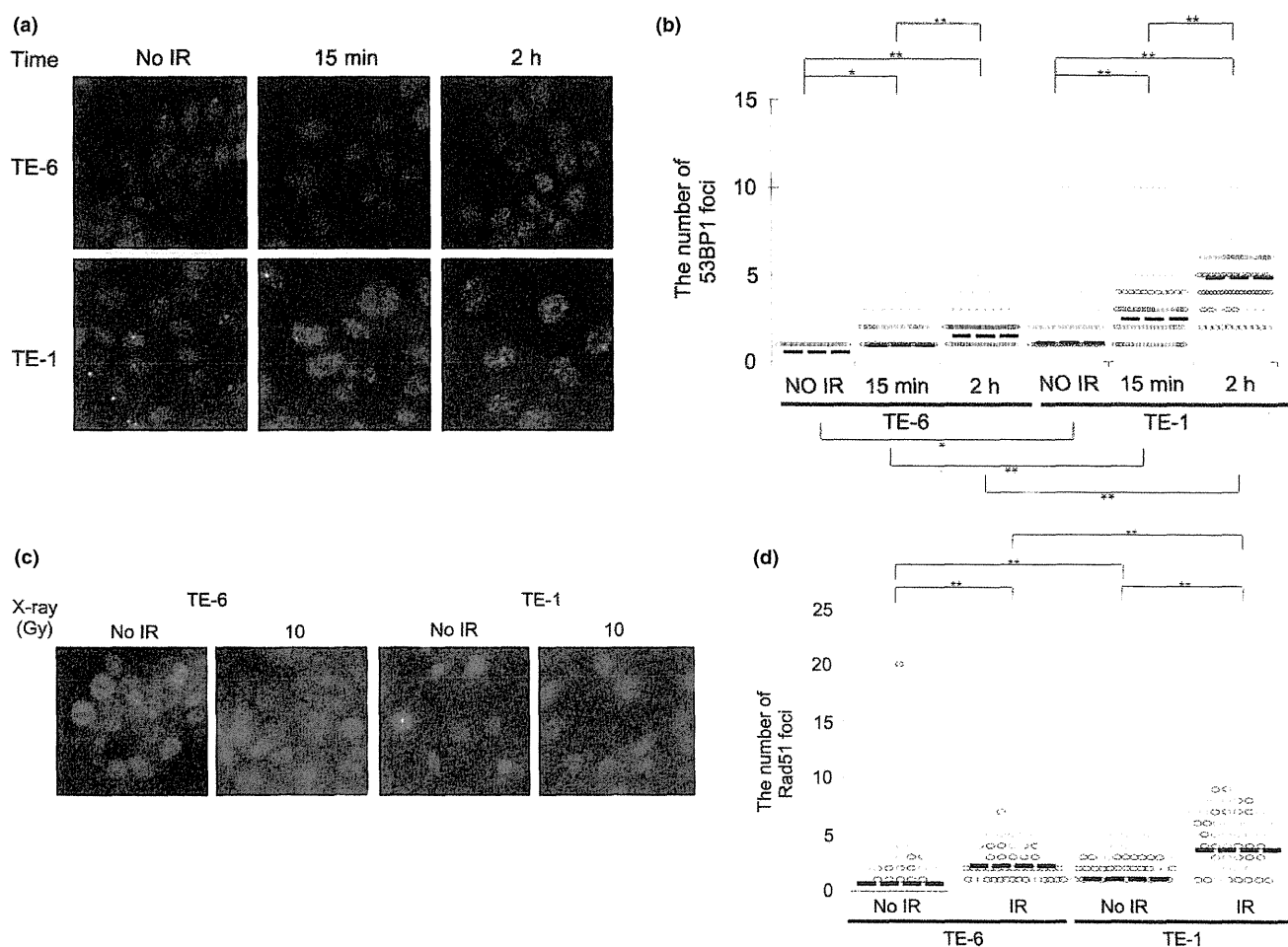


Fig. 6. Impairment of 53BP1 and RAD51 nuclear focus formation in X-ray irradiated TE-6 cells. (a) TE-6 and TE-1 cells were irradiated with 2 Gy of X-rays and fixed 15 min and 2 h after irradiation. The formation of 53BP1 nuclear foci was evaluated by immunofluorescence. (b) Scatter diagrams showing the number of 53BP1 foci in individual cells. The lines shown indicate the mean of the data plotted. The data were obtained from at least 100 cells for each condition. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). (c) TE-6 and TE-1 cells were irradiated with 10 Gy of X-rays and fixed 6 h after irradiation. The formation of RAD51 nuclear foci was evaluated by immunofluorescence. (d) Scatter diagrams showing the number of RAD51 foci in individual cells. The lines shown indicate the mean of the data plotted. The data were obtained from at least 100 cells for each condition. ** $P < 0.01$ (Student's *t*-test).

Table 3. Mutations in the DNA repair-related genes uniquely identified in the exome of TE-6 cells

Novel mutations of DNA repair involved genes of TE-6						
Gene	Chr.	Position	Base change	Variant frequency (%)	Amino acid alteration	PolyPhen2 prediction
<i>RNF8</i>	6	37349032	C>T	100	T448M	Probably damaging
<i>CHAF1A</i>	19	4429530	G>A	87	R567Q	Probably damaging
<i>CEP164</i>	11	117222670	A>G	88.9	D120G	Probably damaging
<i>CLSPN</i>	1	36217027	C>T	19.4	A618T	Benign
<i>POLK</i>	5	74865291	A>G	55	I128V	Benign
<i>ATRX</i>	X	76952184	G>A	100	S84L	Benign

targeting γ -H2AX that accumulates at DNA DSBs and recruits repair proteins, including 53BP1 and RAD51.^(33–35) It was reported that *RNF8*^{-/-} *p53*^{-/-} mice had increased levels of

genomic instability and a remarkably elevated tumor incidence compared to *p53*^{-/-} mice,⁽³⁶⁾ that the knockdown of RNF8 sensitized cells to ionizing radiation, and that disruption of the RING domains of RNF8 impaired DSB-associated ubiquitylation and inhibited retention of 53BP1 and BRCA1 at the DSBs sites.⁽³³⁾ The PolyPhen-2 program predicts a probable deleterious impact of the T448M substitution on the structure and function of RNF8. Although the effect of the T448M mutation on the polyubiquitination ability of RNF8 has not yet been confirmed, the observation that the increase in ubiquitination after X-ray irradiation in the TE-6 cells was less than that of the TE-1 cells suggests that RNF8 is impaired in the TE-6 cells. We hypothesized that this mutation might affect the polyubiquitination ability of RNF8 and, as a result, contribute to the DSB repair defect to some extent. The impact of the mutation or loss of RNF8 to PARP inhibitor sensitivity has to be further evaluated.

Whether RNF8 is the only factor contributing to TE-6 sensitivity should be carefully considered. The anti-tumor effect of PARP inhibitors is obviously determined by the BRCA1 or BRCA2 status in ovarian and breast cancers.⁽³⁷⁾ Meanwhile, the sensitivity of the TE-series cells to AZD2281 is altered by

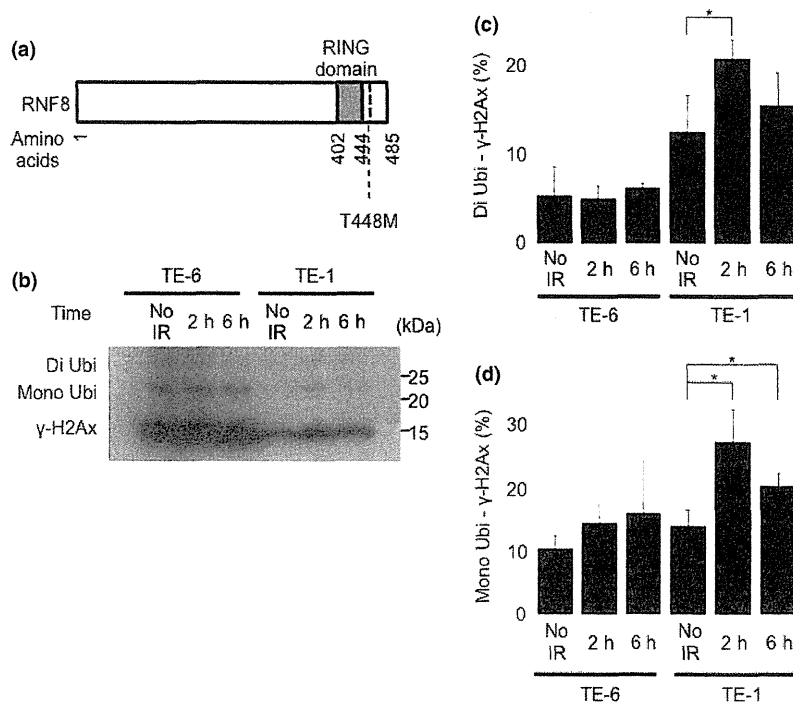


Fig. 7. A novel mutation in the RNF8 gene and the reduced ability of TE-6 cells to polyubiquitinate γ -H2AX. (a) The structure of RNF8 protein and a newly identified T448M amino acid-substitution mutation. (b) Western blot analysis of γ -H2Ax in TE-6 and TE-1 cells treated with 2 Gy of X-rays. (c) The proportion of diubiquitinated γ -H2Ax standardized to non-ubiquitinated γ -H2Ax. The data represent the averages and standard deviations of three independent experiments. (d) The proportion of monoubiquitinated γ -H2Ax standardized to non-ubiquitinated γ -H2Ax. The data represent the averages and standard deviations of three independent experiments.

gradation and most cells exhibited intermediate sensitivity. It may suggest that the AZD2281 sensitivity was regulated by multiple molecular mechanisms in each cell line rather than a single molecule. This multiplicity might make it difficult to identify the distinctive genomic biomarkers of PARP inhibitor sensitivity in ESCC.

We noticed a strong correlation between AZD2281 sensitivity and γ -H2Ax levels in the cells without treatment; this effect might represent the extent of baseline DNA damage. Double strand breaks regularly occur in proliferating cells because topoisomerases bind to DNA and cut the phosphate backbone of the DNA during DNA replication. Though these DSBs are properly repaired in non-tumor cells, it is plausible that unrepaired DNA remains in tumor cells with impaired DSB repair, such as the TE-6 cells. Once a correlation between the baseline level of γ -H2Ax expression and DSB

repair defect is further confirmed, the amount of γ -H2Ax in a tumor tissue sample could be a reasonable biomarker for selecting preferable patients for PARP inhibitor therapy. Further clinical and biological studies of this effect are warranted.

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Disclosure Statement

The authors have no conflict of interest.

References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
- Jamieson GG, Mathew G, Ludemann R, Wayman J, Myers JC, Devitt PG. Postoperative mortality following oesophagectomy and problems in reporting its rate. *Br J Surg* 2004; **91**: 943–7.
- Müller JM, Erasmi H, Stelzner M, Zieren U, Pichlmaier H. Surgical therapy of oesophageal carcinoma. *Br J Surg* 1990; **77**: 845–57.
- Zhang X, Watson DI, Jamieson GG, Lally C, Bessell JR, Devitt PG. Outcome of oesophagectomy for adenocarcinoma of the oesophagus and oesophago-gastric junction. *ANZ J Surg* 2005; **75**: 513–9.
- Crumley AB, Going JJ, McEwan K *et al.* Endoscopic mucosal resection for gastroesophageal cancer in a U.K. population. Long-term follow-up of a consecutive series. *Surg Endosc* 2011; **25**: 543–8.
- Lewis JJ, Rubenstein JH, Singal AG, Elmunzer BJ, Kwon RS, Piraka CR. Factors associated with esophageal stricture formation after endoscopic mucosal resection for neoplastic Barrett's esophagus. *Gastrointest Endosc* 2011; **74**: 753–60.
- Fujishiro M, Yahagi N, Kakushima N *et al.* Endoscopic submucosal dissection of esophageal squamous cell neoplasms. *Clin Gastroenterol Hepatol* 2006; **4**: 688–94.
- Herskovic A, Martz K, al-Sarraf M *et al.* Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *N Engl J Med* 1992; **326**: 1593–8.
- al-Sarraf M, Martz K, Herskovic A *et al.* Progress report of combined chemoradiotherapy versus radiotherapy alone in patients with esophageal cancer: an intergroup study. *J Clin Oncol* 1997; **15**: 277–84.
- Ame JC, Spelnhauer C, de Murcia G. The PARP superfamily. *BioEssays* 2004; **26**: 882–93.
- Gallmeier E, Kern SE. Absence of specific cell killing of the BRCA2-deficient human cancer cell line CAPAN1 by poly(ADP-ribose) polymerase inhibition. *Cancer Biol Ther* 2005; **4**: 703–6.
- Bryant HE, Schultz N, Thomas HD *et al.* Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005; **434**: 913–7.
- Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 2002; **108**: 171–82.

- 14 Weston VJ, Oldreive CE, Skowronska A *et al.* The PARP inhibitor olaparib induces significant killing of ATM-deficient lymphoid tumor cells in vitro and in vivo. *Blood* 2010; **116**: 4578–87.
- 15 Williamson CT, Muzik H, Turhan AG *et al.* ATM deficiency sensitizes mantle cell lymphoma cells to poly(ADP-ribose) polymerase-1 inhibitors. *Mol Cancer Ther* 2010; **9**: 347–57.
- 16 Dedes KJ, Wetterskog D, Mendes-Pereira AM *et al.* PTEN deficiency in endometrioid endometrial adenocarcinomas predicts sensitivity to PARP inhibitors. *Sci Transl Med* 2010; **2**: 53–75.
- 17 McEllin B, Camacho CV, Mukherjee B *et al.* PTEN loss compromises homologous recombination repair in astrocytes: implications for glioblastoma therapy with temozolomide or poly(ADP-ribose) polymerase inhibitors. *Cancer Res* 2010; **70**: 5457–64.
- 18 Wiltshire TD, Lovejoy CA, Wang T, Xia F, O'Connor MJ, Cortez D. Sensitivity to poly(ADP-ribose) polymerase (PARP) inhibition identifies ubiquitin-specific peptidase 11 (USP11) as a regulator of DNA double-strand break repair. *J Biol Chem* 2010; **285**: 14565–71.
- 19 Akbari MR, Malekzadeh R, Lepage P *et al.* Mutations in Fanconi anemia genes and the risk of esophageal cancer. *Hum Genet* 2011; **129**: 573–82.
- 20 Rosenberg PS, Alter BP, Ebell W. Cancer risks in Fanconi anemia: findings from the German Fanconi Anemia Registry. *Haematologica* 2008; **93**: 511–7.
- 21 Stransky N, Egloff AM, Tward AD *et al.* The mutational landscape of head and neck squamous cell carcinoma. *Science* 2011; **333**: 1157–60.
- 22 Drew Y, Mulligan EA, Vong WT *et al.* Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2. *J Natl Cancer Inst* 2011; **103**: 334–46.
- 23 Sakai W, Swisher EM, Karlan BY *et al.* Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 2008; **451**: 1116–20.
- 24 Rodrigue A, Lafrance M, Gauthier MC *et al.* Interplay between human DNA repair proteins at a unique double-strand break in vivo. *EMBO J* 2006; **25**: 222–31.
- 25 Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1, an activator of ATM in response to DNA damage. *DNA Repair (Amst)* 2004; **3**: 945–52.
- 26 Baumann P, West SC. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. *Trends Biochem Sci* 1998; **23**: 247–51.
- 27 Farmer H, McCabe N, Lord CJ *et al.* Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005; **434**: 917–21.
- 28 Bunting SF, Callén E, Wong N *et al.* 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* 2010; **141**: 243–54.
- 29 McCabe N, Turner NC, Lord CJ *et al.* Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res* 2006; **66**: 8109–15.
- 30 Mendes-Pereira AM, Martin SA, Brough R *et al.* Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. *EMBO Mol Med* 2009; **1**: 315–22.
- 31 Turner NC, Ashworth A. Biomarkers of PARP inhibitor sensitivity. *Breast Cancer Res Treat* 2011; **127**: 283–6.
- 32 Wang X, Weaver DT. The ups and downs of DNA repair biomarkers for PARP inhibitor therapies. *Am J Cancer Res* 2011; **1**: 301–27.
- 33 Mailand N, Bekker-Jensen S, Fastrup H *et al.* RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell* 2007; **131**: 887–900.
- 34 Bekker-Jensen S, Mailand N. Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair (Amst)* 2010; **9**: 1219–28.
- 35 Lu CS, Truong LN, Aslanian A *et al.* The RING finger protein RNF8 ubiquitinates Nbs1 to promote DNA double-strand break repair by homologous recombination. *J Biol Chem* 2012; **287**: 43984–94.
- 36 Halaby MJ, Hakem A, Li L *et al.* Synergistic interaction of Rnf8 and p53 in the protection against genomic instability and tumorigenesis. *PLoS Genet* 2013; **9**: e1003259.
- 37 Fong PC, Boss DS, Yap TA *et al.* Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 2009; **361**: 123–34.

Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and methods.

Fig. S1. Effect of AZD2281 on the colony formation of TE-8, TE-10, TE-4, TE-11, TE-14, TE-9 and HCC1937 cells.

Fig. S2. Effect of BSI-201 on the colony formation of TE-1 and TE-6 cells.

Fig. S3. DNA ploidy of TE-1 and TE-6 cells treated with AZD2281 for 12 h.

Fig. S4. Expression of the BRCA1 and BRCA2 proteins in TE-series cells.

Fig. S5. Expression of the 53BP1 and RAD51 proteins in TE-1 and TE-6 cells.

Table S1. Summary of the whole exome sequencing data of TE-1 cells.

Table S2. Summary of the whole exome sequencing data of TE-6 cells.

Identification and Characterization of Cancer Mutations in Japanese Lung Adenocarcinoma without Sequencing of Normal Tissue Counterparts

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Abstract

We analyzed whole-exome sequencing data from 97 Japanese lung adenocarcinoma patients and identified several putative cancer-related genes and pathways. Particularly, we observed that cancer-related mutation patterns were significantly different between different ethnic groups. As previously reported, mutations in the EGFR gene were characteristic to Japanese, while those in the KRAS gene were more frequent in Caucasians. Furthermore, during the course of this analysis, we found that cancer-specific somatic mutations can be detected without sequencing normal tissue counterparts. 64% of the germline variants could be excluded using a total of 217 external Japanese exome datasets. We also show that a similar approach may be used for other three ethnic groups, although the discriminative power depends on the ethnic group. We demonstrate that the ATM gene and the PAPA2 gene could be identified as cancer prognosis related genes. By bypassing the sequencing of normal tissue counterparts, this approach provides a useful means of not only reducing the time and cost of sequencing but also analyzing archive samples, for which normal tissue counterparts are not available.

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Introduction

The advent of next generation sequencing technology has greatly facilitated the detection and characterization of genetic variations in the human genome. Most remarkably, this type of study has driven the 1000 Genomes Project [1,2], which aims to provide a comprehensive map of human genetic variants across various ethnic backgrounds. However, because whole-genome sequencing is still costly, the sequencing of whole exon regions using hybridization capture methods (exome sequencing) [3-5] is widely used to screen for genes that are related to hereditary diseases. By sequencing exomes from healthy and diseased individuals and comparing them, genes that are responsible for many diseases have been identified [6], including Miller syndrome [7,8] and familial hyperkalemic hypertension [9]. Along with the progress that has been made in exome sequencing, the volume of germline single nucleotide

polymorphism (SNP) data that has been registered in dbSNP is rapidly expanding for various populations [10].

Exome sequencing provides a powerful tool for cancer studies as well. Indeed, a number of papers have been published describing the identification and characterization of single nucleotide variants (SNVs) that somatically occur in cancers and are suspected to be responsible for carcinogenesis and disease development [11]. The International Cancer Genome Consortium (ICGC) has been collecting exome data for somatic SNVs that are present in more than 50 types of cancers as a part of an international collaborative effort [12-14]. The Cancer Genome Atlas (TCGA) has developed a large genomic dataset, including exomes for high-grade ovarian carcinoma, that has been used to detect significantly mutated genes, including TP53, BRCA1 and BRCA2 [15]. They have also identified various genomic aberrations and deregulated pathways that may act as therapeutic targets.

In most ongoing cancer exome studies, normal tissue counterparts have been sequenced in parallel with cancer tissue [15-19]. This is assumed to be necessary because germline variants must be excluded from the full set of SNVs to detect the somatic SNVs that are unique to cancers. However, the sequencing of normal tissue counterparts increases the cost and time of the analysis. Also, in some cases, it is difficult to obtain normal tissue counterparts. In addition, it remains unclear how accurately germline SNVs can be excluded using normal tissue exomes. To conservatively exclude germline SNVs, their sequence depths and accuracies may need to be greater than those that are obtained from the cancer exomes.

In this study, we generated and analyzed 97 cancer exomes from Japanese lung adenocarcinoma patients. We also demonstrate that somatic SNVs can be enriched to a level that is sufficient for further statistical analyses even in the absence of the sequencing of normal tissue counterparts. To separate the germline from the somatic SNVs, we first compared the variation patterns between a cancer exome with the 96 other patients' normal tissue exomes. We also attempted to conduct a similar mutual comparison solely utilizing cancer exomes, without the consideration of exomes of normal tissue counterparts. It is true that if we completely omitted normal tissue sequencing, we would tentatively disregard of somatic mutations that occurs at exactly the same genomic position in multiple cancers. However, recent papers have elucidated that such shared SNVs are very rare [15,20-22]. Moreover, many of these recursively mutations have been registered in the cancer somatic mutation databases such as Sanger COSMIC [23,24], and those recurrent SNVs can be recovered by follow-up studies partially using the data from the normal tissues. To understand the unique nature of each cancer, a statistical analysis of the distinct SNVs is presumed to be essential in addition to the analysis of the common SNVs.

In this study, we demonstrate that it is possible to identify the first candidates for cancer-related genes and pathways, even without the sequencing of a normal tissue counterpart. We show that this approach is useful not only to reduce the cost of the sequencing but also to improve the fidelity of the data. It should be also useful for analyzing old archive samples, for which normal tissue counterparts are not always available. Here, we describe a practical and cost-effective method to expedite cancer exome sequencing.

Results and Discussion

Characterization of SNVs using the 97 exome dataset

Firstly, we generated and analyzed whole-exome sequences from 97 Japanese lung adenocarcinoma patients. Exome data were collected from both cancer and normal-tissue counterparts, separated by laser capture microdissection. We purified the exonic DNA (exomes) and generated 76-base paired-end reads using the illumina GAIIX platform. Approximately 30 million mapped sequences were obtained from each sample, providing 74× coverage of the target regions; 93% of the target regions had 5× coverage (Figure S1 in File S1). Burrows-Wheeler Aligner (BWA) [25] and the Genome Analysis Toolkit (GATK) [26,27] were used to identify

SNVs (Figure S2 in File S1). Only SNVs that were detected in cancer tissues and showed no evidence of variation in normal tissues were selected for further analysis.

The obtained dataset was used to characterize the cancer-specific mutation patterns (Table S3 in File S1). We calculated the enrichment of the SNVs within particular genes, protein domains, functional categories, and pathways. We searched for genes with somatic SNVs significantly enriched in Japanese lung adenocarcinoma. As shown in Table S4 in File S1, several genes were identified as significantly mutated. In particular, we searched for domains that are enriched with SNVs and harbor known cancer-related mutations in the COSMIC database. In total, 11 genes were identified ($P < 0.02$, Table 1). For example, the Dbl homology (DH) domain of PREX1 gene [28] was enriched with SNVs ($P = 0.00071$). However, in the PREX2 gene [29], the Pleckstrin homology (PH) domain was enriched with SNVs ($P = 0.011$) (Figure 1A and B). Both the PREX1 and the PREX2 genes activate the exchange of GDP to GTP for the Rho family of GTPases and the DH/PH domains are indispensable for nucleotide exchange of GTPases and its regulation [30-32]. In addition, we analyzed the expression patterns of these genes using a cancer gene expression database, GeneLogic (Figure S3 in File S1). Expression levels of PREX1 and PREX2 were not enhanced in lung adenocarcinoma but were enhanced in wide variety of cancers, which is partly indicated in previous studies [33]. The SNVs in the PREX1 and PREX2 genes, which were concentrated at its pivotal signaling domains, might enhance activities in these genes, and thereby functionally mimics the increased expressions of this gene in some different types of cancers. The cancer-related gene candidates identified from this dataset are listed in Table 1.

Similarly, pathway enrichment analyses using the KEGG database [34] also detected several putative cancer-related pathways. The identified pathways are listed in Table 2. Interestingly, the endometrial cancer pathway [35] was detected in this enrichment analysis ($P = 3.1e-15$, Figure 2A). This pathway includes major cancer-related pathways, for example, the MAPK signaling pathway and the PI3K/AKT pathway. For this pathway, we compared mutation patterns between our Japanese data and those of the previous study of lung adenocarcinoma in Caucasians [21]. We found that the SNVs in the EGFR gene were four times more frequent in the Japanese population than among Caucasian populations (Figure 2B, left panel). EGFR mutations were frequently occurring in non-smoker, female and Asian patients of lung adenocarcinoma [36], which is a molecular target of anti-cancer drug, *gefitinib* [20,37,38]. Conversely, KRAS mutations, which are also well-known cancer-related mutations [39], were more than four times frequent among Caucasians (Figure 2B, center panel). However not all mutational patterns are different between populations. For instance, TP53 harbored mutations in both datasets with similar frequency (Figure 2B, right panel).

Ambiguity in SNV identification of normal tissue counterparts

In the aforementioned analysis, we discriminated germline variants using the normal tissue counterparts. A number of

Table 1. List of the identified possible cancer-related genes.

Gene	Domain	Number of SNVs		
		Domain	Gene	P-value*
EGFR†	IPR001245:Serine-threonine/tyrosine-protein kinase	34	37	4.4e-21
KRAS†	IPR001806:Ras GTPase	6	7	8.0e-6
TNN	IPR003961:Fibronectin, type III	4	5	5.2e-5
TP53†	IPR008967:p53-like transcription factor, DNA-binding	20	23	9.5e-5
PREX1	IPR000219:Dbl homology (DH) domain	4	5	0.00071
DNAH7	IPR004273:Dynein heavy chain	5	7	0.0025
FSTL5	IPR011044:Quinoprotein amine dehydrogenase, beta chain-like	7	7	0.0043
NRXN3	IPR008985:Concanavalin A-like lectin/glucanase	5	7	0.0063
PREX2	IPR001849:Pleckstrin homology	3	7	0.011
FER1L6	IPR008973:C2 calcium/lipid-binding domain, CaLB	3	6	0.013
COL22A	IPR008985:Concanavalin A-like lectin/glucanase	3	6	0.015

* $P < 0.02$

† Reported in the Cancer Gene Census [11]. Note that the genes atop the list are previously reported to be associated with this cancer type, while most of them are novel possible cancer-related genes.

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SNVs initially identified as somatic were also found to be present in normal tissues, thus, were false positive calls under the validations by visual inspection of the mapped sequences and Sanger sequencing. To examine the cause of this problem, we inspected the errors in randomly selected 26 cancers and their normal tissues. On average in each cancer, twenty-five percent of somatic SNV candidates were found to be false positive (Figure 3). In these cases, the sequence coverage and quality of the normal counterpart were not sufficient. Indeed, the sequences supporting each SNV and these qualities were significantly diverged between the cancer and normal tissues. Although we increased the total number of reads in the normal tissues, it was difficult in practice to cover all of the genomic positions (Figure S4 in File S1). A summary of the germline SNV validations is shown in Table S5 in File S1.

However, we noticed that some were correctly identified as germline SNVs in external reference exomes. Twenty-five exomes allowed us to exclude eight false positive calls in each cancer. This raised the possibility that the SNVs from the other patients may be used as surrogates to increase the depth and quality of the sequencing.

Excluding germline SNVs by considering mutual overlaps of other persons' exomes

To further test this possibility, we examined whether cancer exome analyses would be possible without sequencing of the normal tissue counterpart of each cancer. First, we evaluated the extent to which the germline SNVs could be discriminated using external exomes. For this purpose, we used the 97

paired cancer-normal exome datasets for the validation dataset. We found that we could detect 54% of the germline SNVs by using the 96 normal tissue exomes from the external reference (Figure 4A). We further expanded the filtration dataset using the externally available 73 Japanese exome data and 48 in-house Japanese exome datasets. Altogether, we were able to remove 64% of the germline SNVs, using a total of 217 Japanese exome datasets from other individuals, without sequencing each cancer's normal counterpart (Figure 4A). The extrapolation of the graph also indicated that 1,350 and 2,000 samples would be required to remove 90% and 95% of the germline SNVs, respectively. We expect that such a sample size will be available in near future considering current rapid expansion of the exome analysis.

We further evaluated if the same filtration could be done by solely using cancer exomes. We obtained essentially the same results (Figure S5 in File S1). Obvious caveat of this approach is that this would disregard about 3% of somatic SNVs recurrently occurring (Figure S5 in File S1, blue). However, as aforementioned, we found that those recurrent SNVs were very rare [15,19] and most of them were derived from dubious somatic SNVs, which were overlooked in the normal tissues. We also consider that most of those recurrent SNVs, if any, can be analyzed separately by sequencing a limited number of normal tissues.

Filtering out germline SNVs by considering mutual overlaps for different ethnic groups and for rare SNPs

We examined whether SNVs in other ethnic backgrounds could be used as external datasets for the filtration. We obtained exome data from individuals of various ethnic backgrounds from the 1000 Genome Project. We used these exome datasets to exclude the germline SNVs that were identified in the Japanese cancers. We found that the discriminative power was significantly lower compared with exomes from Japanese populations. Therefore, these datasets were not suitable for this purpose (Figure 4B). We also examined and found that the exomes in each ethnic group were useful to discriminate the germline SNVs in the corresponding group (Figure S6, S7 and Table S6 in File S1).

We, then, examined to what extent minor germline variants could be covered with this approach in the Japanese population. We evaluated the sensitivity of the filtration process for the SNVs in the 97 cancers (Figure S8 in File S1). We found that 88% of the germline SNVs occurring in more than five percent of the 97 exomes could be detected using the 73 external Japanese datasets. For the SNVs occurring in 1% of the 97 cancers, 19% could be excluded.

Using the crude dataset to characterize cancer related SNVs and pathways

Taken together, with 217 Japanese exomes used for filtration, 36% of the germline SNVs remained unfiltered. Nevertheless, we considered that it may be still possible to use the crude SNV dataset as a first approximation for identifying and analyzing cancer-related genes and pathway candidates. To validate this idea, we compared the results of enrichment analyses between the crude dataset and the refined somatic

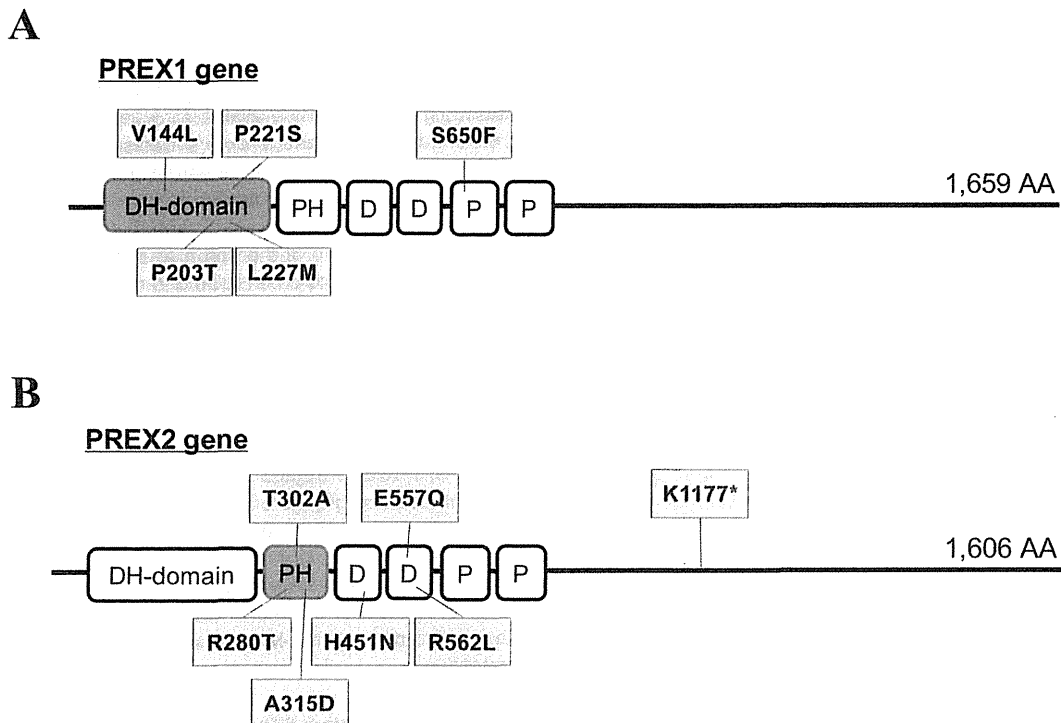


Fig. 1

Figure 1. Identification and characterization of the putative cancer-related genes using 97 cancer exomes. SNVs in the PREX1 (A) and PREX2 (B) genes are represented in the boxes. The protein domains in which the enrichments of the SNVs were statistically significant are represented in orange boxes (also see Materials and Method). DH-domain: Dbl homology (DH) domain; PH: Pleckstrin homology domain; D: DEP domain; P: PDZ/DHR/GLGF.

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SNV datasets, which were generated from the paired cancer-normal exomes.

Most of the putative cancer-related genes and pathways that were identified from the refined dataset were also present in the crude dataset (Tables S7 and S8 in File S1). The example of the TNN gene, which was reported as a marker of tumor stroma [40-42], is shown in Figure S9 in File S1. In this case, even with the germline SNVs, which were unfiltered in the crude dataset (indicated by black in Figure S9 in File S1), the enrichment of somatic SNVs in this domain was statistically significant. In total, nine genes which identified as possessing cancer-related SNVs from the refined dataset were also detected in the crude dataset. On the other hand, two genes from the refined dataset were not represented in the crude dataset. In the pathway analysis, we identified 26 cancer-related pathways which were identified from the refined

dataset. In addition, 19 pathways were also represented in the crude dataset as well as the refined dataset. The overlap between the datasets is summarized in Table 3. It should be noted that statistically enrichment analyses were possible even at the current coverage of the filter dataset. With the expanded external dataset, it would be more practical to subject the candidates to the results of Sanger sequencing validations as well as removing remaining germline SNVs.

Identification of prognosis related genes by using the crude dataset

As one of the most important objectives of the cancer exome studies, we investigated whether mutations affecting cancer prognoses can be identified by using crude dataset (Table S9 and Figure S10 in File S1). In the Kaplan-Meier analysis, seven patients who carried SNVs in the ATM gene (Figure 5A)

Table 2. List of the identified possible cancer-related pathways.

KEGG ID	Pathway definition	Number of cancers with	
		SNVs	P-value*
hsa05213	Endometrial cancer	72	3.1e-15
hsa04320	Dorso-ventral axis formation	48	4.4e-15
hsa05219	Bladder cancer	62	4.9e-14
hsa05223	Non-small cell lung cancer	66	7.1e-12
hsa05214	Glioma	70	6.5e-11
hsa05218	Melanoma	70	1.3e-9
hsa05212	Pancreatic cancer	68	6.9e-9
hsa05215	Prostate cancer	71	4.3e-7
hsa05216	Thyroid cancer	36	1.1e-6
hsa04520	Adherens junction	59	3.7e-6
hsa05210	Colorectal cancer	53	1.8e-5
hsa04012	ErbB signaling pathway	64	2.6e-5
hsa05120	Epithelial cell signaling in <i>Helicobacter pylori</i> infection	53	4.8e-5
hsa04540	Gap junction	60	0.00024
hsa04912	GnRH signaling pathway	61	0.0011
hsa05217	Basal cell carcinoma	41	0.0020
hsa05222	Small cell lung cancer	52	0.0069
hsa05220	Chronic myeloid leukemia	46	0.010
hsa05160	Hepatitis C	67	0.012
hsa05014	Amyotrophic lateral sclerosis (ALS)	36	0.014
hsa04977	Vitamin digestion and absorption	20	0.015
hsa05416	Viral myocarditis	40	0.028
hsa04512	ECM-receptor interaction	47	0.034
hsa02010	ABC transporters	29	0.035
hsa04510	Focal adhesion	78	0.037
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	40	0.039

* $P < 0.05$

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showed statistically significant poor prognoses ($P = 9.6e-6$, Figure 5B). Three SNVs in the ATM gene were significantly enriched in the the phosphatidylinositol 3-/4-kinase catalytic domain ($P = 0.014$). ATM senses DNA damage and phosphorylates TP53, which, in turn, invokes various cellular responses, such as DNA repair, growth arrest and apoptosis, and collectively prevents cancer progression (Figure S11 in File S1) [43,44].

We also examined whether other frequently mutated genes were associated with better or worse prognoses. We found that patients with PAPA2 mutations showed prolonged survival times ($P = 0.026$, Figure 5C and D). PAPA2 proteolyzes IGFBP5 [45,46], which is an inhibitory factor for IGFs [47]. Mutations in the PAPA2 gene may result in the accumulation of IGFBP5, and the resulting decrease in IGF signaling may impair the proliferation of cancer cells [48]. Again, it should be noted that for both the ATM and PAPA2 genes, the statistical significance of the prognostic difference persisted both before (black line) and after (red line) the remaining germline

mutations were removed, which was validated by Sanger sequencing (Figure 5B, D and Table S10 in File S1).

Conclusions

We have identified and characterized the SNVs in lung adenocarcinoma in a Japanese population. Further biological evaluations of the discovered SNVs will be described elsewhere. In particular, information of transcriptome and epigenome should be important for further analyses of cancer genomes, as they would shed new lights on the cancer biology (Table S1) [49]. In this study, we also presented a useful approach for the analysis of cancer exomes, without the need to sequence the normal tissue counterpart. We believe that the approach not only lowers the barriers in cost, time and data fidelity in the exome analysis, but also enables exome analysis of archive samples, for which normal tissue counterparts are not always available.

Materials and Methods

Ethics statement

All of the samples were collected by following the protocol (and written informed consent) which were approved by Ethical Committee in National Cancer Center, Japan (Correspondence to: Katsuya Tsuchihara; ktsuchi@east.ncc.go.jp).

Case selection and DNA preparation

All of the tissue materials were obtained from Japanese lung adenocarcinoma patients with the appropriate informed consent. Surgically resected primary lung adenocarcinoma samples with lengthwise dimensions in excess of 3 cm were selected. Data on the 52 patients who had relapses and other clinical information about the 97 cases are shown in Table S11 in File S1. All 97 cancer and normal tissues were extracted from methanol-fixed samples by laser capture microdissection. DNA purification was performed using an EZ1 Advanced XL Robotic workstation with EZ1 DNA Tissue Kits (Qiagen).

Whole-exome sequencing

Using 1 μ g of isolated DNA, we prepared exome-sequencing libraries using the SureSelect Target Enrichment System (Agilent Technologies) according to the manufacturer's protocol. The captured DNA was sequenced by the illumina Genome Analyzer Ix platform (Illumina), yielding 76-base paired-end reads.

Somatic SNV detection

The methods that were used to detect the SNVs, including BWA, SAMtools [50] and GATK, are shown in Figure S2 in File S1. Using data from NCBI dbSNP build 132 and one Japanese genome [51], major germline SNVs were excluded. In addition, rare germline SNVs were discarded using 97 exomes from normal tissue counterparts, 73 Japanese exomes provided from the 1000 Genomes Project (the phase1 exome data, 20110521) and 48 in-house Japanese exomes. We also validated a portion of the SNV datasets by the Sanger