Heliyon 9 (2023) e22082

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Enhancing tumour content and tumour cell count using microdissection contributes to higher detection rate of genetic mutations by next-generation sequencers☆

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ARTICLE INFO

Keywords:
Oncomine Dx target test
Microdissection
Next-generation sequencing
Tumour content
Genetic mutation

ABSTRACT

Background: Next-generation sequencing (NGS) analysis is becoming indispensable for the treatment of advanced lung cancer. NGS analysis requires a large number of cancer cell-containing tissues; however, it is often difficult for small biopsies to obtain the required quantities. In microdissection, only the tumour parts of a tissue specimen are obtained, which thereby increases the tumour content and tumour cell count of the tissue specimen. In this study, we investigated the extent to which the detection rate of genetic mutations changes by increasing the tumour content using microdissection.

Patients and methods: This is a retrospective study. In the genetic panel test using the Oncomine Dx Target Test (ODxTT), participants were divided into two groups: before (group A; April 2021–March 2022) and after (group B; April 2022–December 2022) the introduction of microdissection. The submission criteria for ODxTT were tumour content and tumour cell count >30 % and >2000 in group A, and >40 % and >5000 in group B, respectively. We compared the rate of genetic mutations detected using ODxTT between the two groups.

Results: This study included 214 consecutive ODxTT cases between April 2021 and December 2022. In group A (n = 112), 65 cases were adenocarcinoma, 84 involved lung tissue, and 64 underwent bronchoscopic sampling, whereas in group B (n = 102), 55 cases were adenocarcinoma, 91 cases involved lung tissue, and 79 cases underwent bronchoscopic sampling. Furthermore, genetic mutations were detected in 39 of 112 cases (35 %) in group A and 59 of 102 cases (58 %) in group B, which was statistically higher in group B (P = 0.0006). Genetic mutations were detected in 45 of 55 adenocarcinoma cases in group B. The genetic mutations detected in epidermal growth factor rescepor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and mesenchymal epithelial transition (*MET*) were higher in group B.

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https://doi.org/10.1016/j.heliyon.2023.e22082
Received 14 May 2023; Received in revised form 1 November 2023; Accepted 3 November 2023
Available online 7 November 2023

Conclusion: Increasing the number of tumour cells and tumour content can enhance the detection rate of genetic mutations using ODxTT.

1. Introduction

The discovery of actionable gene mutations and the clinical usage of molecularly targeted drugs have improved long-term survival in patients with advanced non-small cell lung cancer (NSCLC). For example, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) and anaplastic lymphoma kinase (ALK) inhibitors have been shown to prolong progression-free survival and overall survival compared to cytotoxic chemotherapy for advanced NSCLC with *EGFR*-activating mutations and *ALK* rearrangement [1–3]. Therefore, the diagnosis of actionable gene mutations is becoming an important issue. In Japan, the use of TKIs for driver mutations requires detection of actionable gene mutations using companion diagnostics (CDx).

Next-generation sequencing (NGS) can detect multiple driver oncogenes simultaneously with limited amounts of tissue samples [4]. The Oncomine Dx Target Test (ODxTT) is the first NGS-based multiplex gene panel test approved by the US Food and Drug Administration, which was approved in Japan in 2019 for the detection of multiple driver mutations [5]. Notably, ODxTT can examine the 46 oncogenic gene mutations, including genetic mutations with molecularly targeted drugs available for lung cancer. The success of NGS analysis is becoming indispensable, as detecting driver genes and treating them using corresponding molecularly targeted drugs prolongs survival greater than that without treatment using molecularly targeted drugs [6].

However, NGS requires a large number of cancer cell-containing tissues [7,8], and it is often difficult for small biopsies to obtain the required quantities at the time of lung cancer diagnosis. According to the Summary of Safety and Effectiveness data, ODxTT requires a DNA concentration of $\geq$0.83 ng/μL and RNA concentration of $\geq$1.43 ng/μL. Furthermore, sufficient concentrations of DNA and RNA can be isolated from tissue sections with a tumour cell content of $\geq$20 % [9].

Microdissection is a technique in which only the tumour parts of a tissue specimen are extracted using an equipment with a laser irradiation device connected to a microscope, which can be used to increase the tumour content and number of tumour cells of the tissue specimen [10]. A high tumour content is desirable for molecular testing as the presence of non-tumour cells can hinder the detection of clinically relevant somatic mutations relative to the detection thresholds of various molecular technologies [10]. Therefore, microdissection may contribute to more accurate genetic information in cancer cells and reduce false negatives by sectioning out targeted cellular regions from formalin-fixed paraffin-embedded sections (FFPE).

In this study, we investigated the usefulness of microdissection in terms of the rate of specimen submission to next-generation sequencers and the frequency of detection of genetic mutations.

2. Materials and methods

2.1. Study design

This was a single-center retrospective cohort study, which was approved by the ethics committee of National Hospital Organization Kinki-Chuo Chest Medical Center (clinical research review board Kinki-Chuo Chest Medical Center: approval number 2022-042), and all the patients gave written informed consent for their tissues to be collected and stored before examing ODxtt. The requirement for written informed consent was waived by the committee because of the retrospective nature of the study. Therefore, the opt-out for this study was available through the hospital website. And also, this study was conducted in accordance with the Declaration of Helsinki.

Patients serially diagnosed with NSCLC using biopsy or surgical samples at the Kinki-Chuo Chest Medical Center between April 2021 and December 2022 were retrospectively evaluated. During this period, ODxTT (Thermo Fisher Scientific, Waltham, MA, USA) was used in preference to singleplex testing when the sample was measurable enough to the number of tumour cells and tumour content by ODxTT. The ODxTT was covered by health insurance. If not measurable by ODxTT due to luck of the number of tumour cells and tumour content, cobas EGFR test and ALK immunohistochemistry were used instead of ODxTT during April 2021 to March 2022, and Amoy, a multiplex testing covered by health insurance, or cobas EGFR and ALK immunohistochemistry were used during April 2022 to December 2022.

Finally, we divided the patients into two groups: before and after the introduction of microdissection. Patients in the group before the introduction of microdissection (from April 2021 to March 2022) were classified as group A, while those in the group after the introduction of microdissection (from April 2022 to December 2022) were classified as group B.

2.2. Data collection

We collected data including specimen sampling methods, location of organs sampled, histological pattern, and the results of ODxTT from the pathology records and electronic medical record system.

2.3. Sample processing and genetic tests

Tumour biopsy samples were immediately placed in 10 % neutral buffered formalin and fixed for 12–24 h at room temperature

(20−27 °C). The formalin-fixed tissues underwent serial processing and paraffin embedding to create formalin-fixed paraffin-embedded tissue blocks.

The number of nucleated cells (tumour and inflammatory cells) in haematoxylin and eosin-stained sections was evaluated by three pathologists to determine the percentage of tumour content. For evaluating the tumour content of the tissue, the average of three sample locations was used. Furthermore, when selecting lesions for evaluation, sites of lymphocytic infiltration, sites with high stromal content, and sites with predominant necrosis were avoided.

In group A, if the tumour cell percentage was less than 30 % and the number of nucleated cells was less than 2000 (this is the criteria recommended in the test submission), the samples did not undergo ODxTT. Similarly, in group B, if the tumour content was less than 40 % and the number of nucleated cells was less than 5,000, the samples did not undergo ODxTT. The digitally guided microdissection 'AVENIO Millisect System' was used to optimise the tumour yield and minimise contamination with precise tissue dissection.[7] An example sample using microdissection is shown in Fig. 1. A total of 15–30 slide sections of small biopsy samples and five slide sections of surgical resection samples were submitted to the SRL Diagnostics laboratory (Tokyo, Japan) for ODxTT testing. If we could not examine the sample due to lack of materials, we could examine only two kinds of gene mutations using the single-plex PCR testing for EGFR testing and using the immunostaining for ALK testing.

2.4. Statistical analysis

We compared the detection rates of genetic mutations before and after microdissection. The data are presented as counts and percentages for the qualitative variables. Fisher's exact test was performed to compare the positivity rates between the two groups. Statistical significance for all analyses was defined as a p-value of <0.05. All statistical analyses were conducted using the JMP 12 software.

3. Results

3.1. Sample data

This study included 214 consecutive cases of ODxTT that were performed between April 2021 and December 2022. Table 1 presents details regarding the sample data. There were 120 cases (56 %) of adenocarcinoma and 70 cases (33 %) of squamous carcinoma. Additionally, 175 cases (82 %) involved the lung tissue and 24 cases (11 %) involved the lymph nodes. Furthermore, 143 cases (67 %) underwent bronchoscopic tissue sampling and 21 cases (10 %) underwent endobronchial ultrasound-guided transbronchial needle aspiration. In addition, after the introduction of microdissection almost all tissues with a diagnosis of cancer could be examined, despite the increased tumour contents.

There were 112 cases in group A and 102 cases in group B. In group A, 65 cases (58 %) were adenocarcinoma, 84 cases (75 %) involved the lung tissue, and 64 cases (57 %) underwent bronchoscopic tissue sampling. Furthermore, in group B, 55 cases (54 %) were adenocarcinoma, 91 cases (89 %) involved the lung tissue, and 79 cases (77 %) underwent bronchoscopic tissue sampling.

3.2. Detection of genetic mutations

3.2.1. Group A

Genetic mutations were detected in 39 of 112 cases (35 %) in group A, including 13 cases (12 %) of *EGFR*, 9 cases (8 %) of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and 5 cases (4 %) of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*). Details are presented in Table 2(a).

In the adenocarcinoma subgroup of group A ($n = 65$), genetic mutations were detected as follows: 13 cases (20 %) of *EGFR*, 9 cases (14 %) of *KRAS*, and 3 cases (5 %) of erb-b2 receptor tyrosine kinase 2 (*ERBB2*) (Table 2(b)). Additionally, in the squamous carcinoma

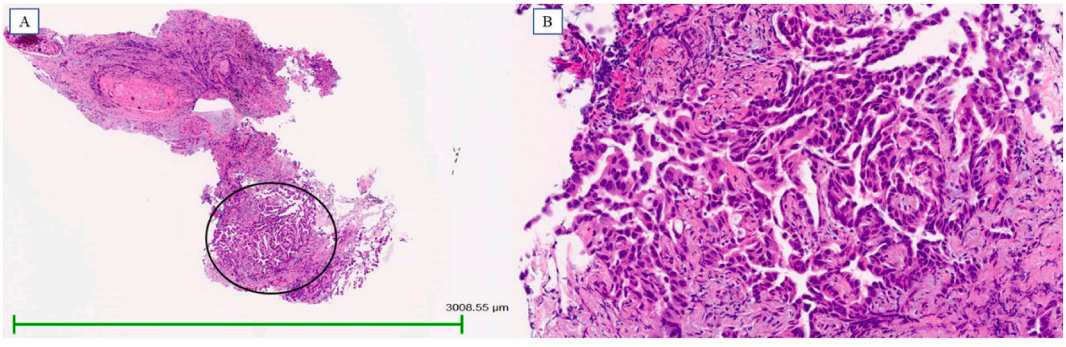

Fig. 1. Fig. 1-a is the figure of tissue specimen with HE staining, and the black circle area is the designated area for ODxTT cutout. Fig. 1-b is the figure of tissue specimen after the designated areas were cut out.

Table 1
Sampling data.

Sample	All cases $n = 214$	Group A $n = 112$	Group B $n = 102$
Histology			
Squamous carcinoma	70	34	36
Adenocarcinoma	120	65	55
Not otherwise specified	24	13	11
Tissue			
Lung	175	84	91
Lymph nodes	24	17	7
Pleural/Pericardial effusion	7	7	0
Pleural	3	2	1
Subcutaneous	1	1	0
Adrenal gland	1	1	0
Brain	2	0	2
Liver	1	0	1
Biopsy methods			
Cell block (Pleural/Pericardial effusion)	7	7	0
Computed tomography-guided needle biopsy	8	4	4
Echocardiography-guided biopsy	3	1	2
Endobronchial ultrasound-guided transbronchial needle aspiration	21	14	7
EUS-FNA	1	1	0
Surgical biopsy	31	21	10
Bronchoscopic biopsy	143	64	79

and not otherwise specified (NOS) subgroup, 5 of 47 cases (11 %) involved *PIK3CA* mutation (Table 2(c)).

3.2.2. Group B

In group B, genetic mutations were detected in 59 of 102 cases (58 %), including 19 cases (19 %) of *EGFR*, 16 cases (16 %) of *KRAS*, 8 cases (8 %) of mesenchymal epithelial transition (*MET*), and 6 cases (6 %) of *ERBB2*. Details are presented in Table 2(a).

In the adenocarcinoma subgroup ($n = 55$), genetic mutations were detected in 45 cases (82 %), including 19 cases (35 %) of *EGFR*, 13 cases (24 %) of *KRAS*, and 4 cases (7 %) each of *MET* and *ERBB2* (Table 2(b)). Additionally, in the squamous carcinoma and NOS subgroup, 4 cases (9 %) were of *MET*, 3 cases (6 %) were of *KRAS*, and 2 cases (4 %) were of *PIK3CA* and *ERBB2* mutations each (Table 2(c)).

4. Discussion

This study exhibited that microdissection can increase the tumour cell count and tumour content, thereby enhancing the detection rate of genetic mutations (before microdissection: 35 %, after microdissection: 58 %, Table 2). Our results indicated that the detection

Table 2
Detection of genetic mutations using the Oncomine Dx Target Test before (group A) and after (group B) the introduction of microdissection (a) Samples with all histology.

Gene mutation	Group A $n = 112$ (%)	Group B $n = 102$ (%)
EGFR	13 (12)	19 (19)
EGFR Del 19+	5 (4)	6 (6)
EGFR L858R	7 (6)	8 (8)
EGFR Uncommon	1 (1)	5 (5)
KRAS	9 (8)	16 (16)
KRAS G12C	4 (4)	4 (4)
KRAS Other	5 (4)	12 (12)
MET	1 (1)	8 (8)
ERBB2	3 (3)	6 (6)
ALK	2 (2)	1 (1)
RET	2 (2)	2 (2)
ROS1	1 (1)	0
BRAF V600E	1 (1)	1 (1)
BRAF Other	1 (1)	1 (1)
PIK3CA	5 (4)	2 (2)
NRAS	0	1 (1)
RAF1	0	1 (1)
IDH2	0	1 (1)
FGFR2	1 (1)	0
None	73 (65)	43 (42)

All values are represented as n (%).

rates of *EGFR* uncommon mutation, *MET* exon 14 skipping mutation, and *KRAS* mutation were elevated after microdissection.

In multi-gene mutation search studies, the percentage of *EGFR* mutations in lung adenocarcinomas among Asians is generally reported to be approximately 50 % [11–13]. In contrast, the percentage of *EGFR* mutations in multi-gene mutation searches using commercial-based ODxTT has been reported to be approximately 20–40 %, clearly indicating a low detection rate of *EGFR* mutations [14,15]. In these reports, the tumour content and the number of tumour cells to be submitted for examination were set at $\geq$ 30 % and $\geq$2,000, respectively, similar to the submission criteria for ODxTT in group A of the present study. Additionally, the detection rate of *EGFR* mutations (20 %) was similar. In our study, increasing the tumour cell count and tumour content using microdissection not only enhanced the detection rate of *EGFR* mutations (35 %) but also that of *KRAS* mutations (24 %) and *MET* exon 14 skipping mutations (7 %). The major possible reason for these results is that microdissection is a highly effective method for enriching the tumour content while preserving the DNA yield. Maximising the tumour burden prior to molecular testing is effective in eliminating potential false negative results. Despite the minimum tumour content percentage of $\geq$20 % required for ODxTT [9], the tumour content percentage may be a risk factor for false negative results when using ODxTT [10]. Another reason is that during the sectioning of small tissues at the time of specimen submission, the tumour content may be different in sections that are submitted at the time of sectioning than in sections that are confirmed for tumour content. Fig. 2 shows the hypothetical changes in the intercept of a small tissue specimen. As hypothesised in the figure, efforts to increase the tumour volume in advance will be important for obtaining sufficient amounts of tumour DNA and RNA for testing.

Ariyasu et al. [16] reported that the feasibility of ODxTT in the clinical setting was relatively lower than that of ALK immunohistochemistry and cobas EGFR. As NGS requires high-quality tumour samples, it is difficult to perform CDx. Therefore, the examination rate of ODxTT (80.2 %) was lower than that of ALK immunohistochemistry (85.0 %) and cobas EGFR (92.8 %). However, using microdissection can overcome this problem. Microdissection has been able to increase the small biopsy specimen submission rate, thereby enhancing the cut-off value of tumour cell count and tumour content for submission. In the study by Geiersbach et al. [10], digitally guided microdissection was conducted on multiple smaller regions having a high tumour content that were chosen from within the larger areas marked for manual microdissection. In this study, samples obtained using digitally guided microdissection exhibited a significantly higher *KRAS* mutant allele fraction and estimated tumour content. Of the 32 samples analysed, 7 (22 %) exhibited a detectable mutation only when digitally guided microdissection was used. These results indicate that digitally guided microdissection can cause a significant enhancement in the tumour content.

In our study, there were differences between groups A and B in the evaluation of specimens and tissue collection methods. In group A, the inability to obtain tumour sections using microdissection often precluded ODxTT testing on smaller biopsy materials, such as specimens collected by bronchoscopy. Therefore, the percentage of ODxTT testing on surgical specimens, needle biopsies, and cell blocks with high tumour components had increased for group A. In contrast, for group B, extracting the tumour portion using microdissection not only successfully enhanced the tumour content and the number of tumour cells, but also allowed a high percentage of small biopsy material to be submitted for ODxTT testing. As the tumours could be purified, it was possible to raise the gene mutation array during ODxTT testing, which we believe led to an increase in the detection rate of genetic mutations in group B. Additionally, small biopsies using bronchoscopy do not have a higher detection rate of genetic mutations than that of other biopsy methods; instead, a higher detection rate can be obtained using surgical specimens. Therefore, in our study, it was important to purify the tumour and enhance the tumour cell count.

This study has some limitations. First, this was a retrospective study conducted at a single institution. Therefore, the methods of specimen sampling, sample processing, and judgement of ODxTT ordering may have been biased. In order to reduce bias in the evaluation of the tumour content in the sample, three different points in the tissue specimen were examined by three pathologists. Validation in consecutive cases reduces the risk of selection bias. Furthermore, specimen submission criteria were clearly defined, and testing methods are constant because they are commercial based. Second, the number of cases in the present study is relatively small to

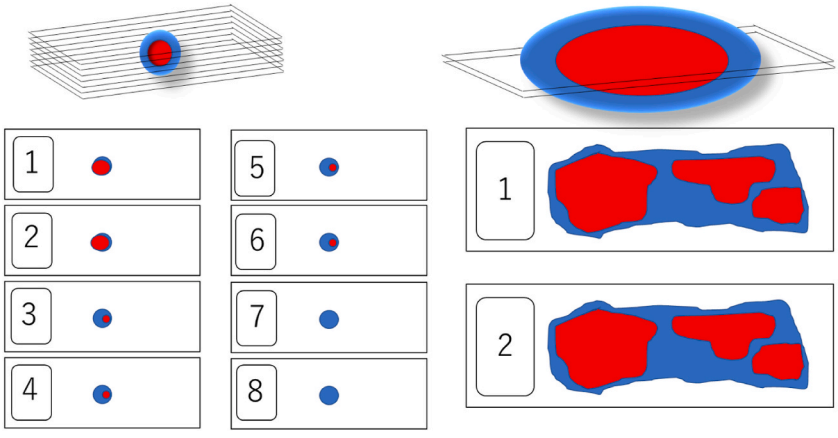

Fig. 2. Hypothetical changes in the intercept of a small tissue specimen. (a) Small biopsy specimen obtained using bronchoscopy. (b) Specimen obtained using surgical biopsy. Red represents tumour areas, whereas blue represents non-tumour areas.

evaluate detective genetic mutation rate compared high tumour content and tumour-cell count with low tumour content and tumour cell count, and for evaluating the usefulness of microdissection. However, the rates of sample submission and detection of genetic mutations were significantly enhanced in this study, despite the small number of cases.

5. Conclusion

This study indicated that increasing the number of tumour cells and tumour content can enhance the detection rate of genetic mutations, especially, EGFR uncommon mutations, KRAS mutations and MET exon 14 skipping, using ODxTT. Moreover, using microdissection for sample processing is beneficial not only for increasing the number of tumour cells and tumour content but also for increasing the rates of sample submission, and, as the results, detection of genetic mutations using ODxTT. In the future, more studies with a larger number of samples were warranted to validate these results.

Contributorship statement

A.T, K.K and S.S conceived the idea of the study. A.T developed the statistical analysis plan and conducted statistical analyses. A.T, K.K, Y.I, Y.T, K.N, K.O, M.T, K.T and S.S contributed to the interpretation of the results. A.T and K.K drafted the original manuscript. Y. M, M.T, T.K and S.S supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically on intellectual content. All authors approved the final version of the manuscript to be published.

Funding

None declared.

Data availability statement

Data will be made available on request.

(b) Adenocarcinoma subgroup

Gene mutation	Group A $n = 65$ (%)	Group B $n = 55$ (%)
EGFR	13 (20)	19 (35)
EGFR Del 19+	5 (8)	6 (11)
EGFR L858R	7 (11)	8 (15)
EGFR Uncommon	1 (2)	5 (9)
KRAS	9 (14)	13 (24)
KRAS G12C	4 (6)	3 (5)
KRAS Other	5 (8)	10 (18)
MET	0	4 (7)
ERBB2	3 (5)	4 (7)
ALK	2 (3)	1 (2)
RET	2 (3)	1 (2)
ROS1	1 (2)	0
BRAF V600E	1 (2)	1 (2)
BRAF Other	1 (2)	1 (2)
PIK3CA	0	0
NRAS	0	1 (2)
RAF1	0	0
IDH2	0	0
FGFR2	0	0
None	33 (51)	10 (18)

All values are represented as n (%).

(c) Squamous carcinoma and not otherwise specified subgroup

Gene mutation	Group A $n = 47$ (%)	Group B $n = 47$ (%)
EGFR	0	0
EGFR Del 19+	0	0

(*continued on next page*)

(*continued*)

Gene mutation	Group A $n = 47$ (%)	Group B $n = 47$ (%)
EGFR L858R	0	0
EGFR Uncommon	0	0
KRAS	0	3 (6)
KRAS G12C	0	1 (2)
KRAS Other	0	2 (4)
MET	1 (2)	4 (9)
ERBB2	0	2 (4)
ALK	0	0
RET	0	1 (2)
ROS1	0	0
BRAF V600E	0	0
BRAF Other	0	0
PIK3CA	5 (11)	2 (4)
NRAS	0	0
RAF1	0	1 (2)
IDH2	0	1 (2)
FGFR2	1 (2)	0
None	40 (85)	33 (70)

CRediT authorship contribution statement

Akihiro Tamiya: Writing – review & editing, Writing – original draft, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kensuke Kanaoka:** Writing – review & editing, Resources, Project administration, Investigation, Data curation, Conceptualization. **Yuji Inagaki:** Writing – review & editing, Project administration, Investigation. **Yoshihiko Taniguchi:** Writing – review & editing, Project administration, Investigation. **Keiko Nakao:** Writing – review & editing, Resources, Project administration. **Yoshinobu Matsuda:** Writing – review & editing, Supervision, Resources, Project administration. **Kyoichi Okishio:** Writing – review & editing, Supervision, Project administration. **Maiko Takeda:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Takahiko Kasai:** Validation, Supervision, Methodology, Data curation. **Shigeki Shigeki:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

All authors have no disclosure inside the submitted work. And, outside the submitted work, Tamiya A received grants from AstraZeneca, Daiichi-Sankyo and Beigene. And received the personal fees from Eli Lilly, Ono Pharmaceutical, Chugai Pharmaceutical, Boehringer Ingelheim, AstraZeneca, Bristol-Myers Squibb, Amgen, Taiho Pharmaceutical, Kyowa Kirin, MSD, Takeda Pharmaceutical, Nihon-Kayaku, Novartis, Thermo Fischer, and Merck BioFarma. Taniguchi Y received personal fees from Chugai Pharmaceutical, Ono Pharmaceutical, and AstraZeneca. Inagaki Y received grants from Osaka Cancer Society, and received personal fees from AstraZeneca, Chugai Pharmaceutical, Chugai-igakusya. Kanaoka K received personal fees from AstraZeneca. Matsuda Y received personal fees from Hisamitsu Pharmaceutical, Fujimoto Pharmaceutical, Takeda Pharmaceutical, Boehringer Ingelheim, and Chugai Pharmaceutical. Okishio K received personal fees from Bristol-Myers Squibb, AstraZeneca, Chugai Pharmaceutical, Nihon-Kayaku, Takeda Pharmaceutical, Taiho Pharmaceutical, Sawai Pharmaceutical. And Shimizu S received personal fees from Takeda Pharmaceutical, Merck BioFarma, Bunkodo, Ishiyaku publishers, inc.

Acknowledgements

We wish to thank the patients who participated in this study. Additionally, we are grateful to the staff of the pathology department for their assistance.

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