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Liquid Biopsy Cell-free DNA Biomarkers in Patients With Oligometastatic Colorectal Cancer Treated by Ablative Radiotherapy

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Abstract. *Background/Aim: To investigate the usefulness of cell-free DNA (cfDNA) in patients with oligometastasis. Patients and Methods: This study included oligometastatic colorectal cancer (CRC) patients who underwent ablative irradiation using stereotactic body radiotherapy or proton beam therapy for metastatic lesions at a single institution. cfDNA was purified from the plasma of pretreated patients and gene mutations were analyzed by next-generation sequencing. Progression-free survival (PFS) was statistically compared according to gene mutation, clonality or allele frequency. Results: A total of 20 patients were analyzed. Mutations were detected in the following genes; TP53 (45%), APC (40%), KRAS (15%), PIK3CA (15%), NF1 (5%), BRCA1 (5%), ERBB2 (5%), FBXW7 (5%), KIT (10%), and HRAS (10%). Patients with multi-clonality of gene mutation showed tendency for poor PFS (p=0.07). Among 7 patients whose metastatic site was the lung, those with no cfDNA detected had significantly better PFS than those with cfDNA (p=0.02). Conclusion: cfDNA profiles could be predictive tools for early recurrence of oligometastatic CRC patients after ablative radiotherapy.*

Systemic malignant disease is generally defined as the existence of distant metastases at the initial diagnosis or after definitive therapy (1). Patients with oligometastases present with a limited number of metastatic tumors at a limited number of sites and have a better prognosis than those with other forms of systemic disease (2). Recent studies have highlighted oligometastatic lesions as therapeutic targets, and a phase II trial revealed that local irradiation can improve the prognosis of affected patients (3). Currently, the ESMO consensus guideline for metastatic colorectal cancer (CRC) recommends local ablative therapy, including stereotactic body radiotherapy (SBRT), for patients with oligometastatic CRC (4). However, more than a few patients have been reported to develop early recurrences after ablative treatments, including SBRT, suggesting that the selection of the most suitable patients for such treatments remains an issue of particular concern (5). The selection of optimal candidates based on diagnostic imaging, including PET-CT, remains challenging. Therefore, studies of potential biomarkers that could identify the optimal candidates for the ablative local treatment of oligometastatic lesions are strongly warranted.

Liquid biopsy techniques involving the analysis of cell-free DNA (cfDNA) in blood samples from patients have been utilized increasingly in clinical practice in recent years (6). Several reports have demonstrated the potential usefulness of cfDNA not only for the early diagnosis of CRC and monitoring of progression after resection surgery, but also for the prognostic prediction of metastatic CRC (7-9). Such reports focused mainly on the validity or usefulness of liquid biopsy in patients with systemic or resectable disease. However, the clinical significance of the cfDNA profile in patients with oligometastatic diseases, including CRC, remains unclear.

In this study, we aimed to use next-generation sequencing to analyze the cfDNA profiles of patients with oligometastatic CRC who received ablative radiotherapy for metastatic lesions. We further aimed to investigate the associations of these cfDNA profiles with clinical outcomes, including early

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recurrences after ablative radiotherapy. Mainly, we focused on patients with CRC because this malignancy provides an optimal target against which to explore the efficacy of local ablative treatment for oligometastatic lesions in terms of the clinical outcomes.

Patients and Methods

Patient selection. This study included oligometastatic CRC patients who underwent ablative radiotherapy using proton beam therapy (PBT) or stereotactic body radiotherapy for metastatic lesions at the National Cancer Center Hospital East in Japan between December 2011 and December 2017. In this study, oligometastatic disease was defined as 1-3 metastases and control of all other lesions, including the primary lesion, by local treatments such as surgical resection. The study protocol was approved by the Research Ethics Committee of our institution (reference number: 2017-487). The research was conducted in accordance with the principles set forth in the 1964 Declaration of Helsinki and its subsequent amendments. All the study participants provided informed, written consent regarding the comprehensive study protocol at our institute before receiving the initial treatment. Data on the patients' pretreatment characteristics and prognoses were collected from medical records.

Sample collection and detection of cfDNA. Plasma samples were obtained from the National Cancer Center Biobank with the approval of the National Cancer Center Biobank management committee. Peripheral venous blood samples were collected into EDTA-containing tubes before patients underwent PBT or SBRT for metastatic lesions and were immediately processed to isolate plasma by centrifugation at 1,600 × *g* and 4˚C for 10 min. Aliquots of the plasma samples were stored at –80˚C.

Next, cfDNA was purified from 4 ml of patient plasma, and fragmented DNA was ligated with adapters to generate libraries. Library construction and sequencing were performed using the Illumina Novaseq 6000 sequencing platform (Illumina, San Diego, CA, USA) with the SureSelect NCC oncopanel v.2 (Agilent, Santa Clara, CA, USA), which can investigate the exons of 114 genes. We obtained 40-45 Gb of FASTA data per sample, aligned the FASTQs to the human genome (hg19), and identified point mutations using Sure call 4.1.1.5 (Agilent). Potential mutations in the following genes were evaluated: *TP53*, *APC*, *KRAS*, *PIK3CA*, *NF1*, *EGFR*, *SMAD4*, *BRAF*, *BRCA2*, *AR*, *PDGFRA*, *BRCA1*, *ERBB2*, *NRAS*, *FBXW7*, *FGFR2*, *KIT*, and *HRAS*. A mutation was defined as "multi-clonal" if the mutant allele frequency was <50% of the maximum allele frequency (MaxAF) in the sample and as "clonal" if the frequency exceeded this threshold.

Statistical analysis. All the statistical analyses were performed using R software, version 3.4.0 (The R Foundation, Vienna, Austria). Progression-free survival (PFS) was defined as the time from the start of radiotherapy until the detection of the earliest signs of disease progression or death from any cause. Hazard ratios (HR) and 95% confidence intervals (CI) were evaluated. Survival curves were constructed using the Kaplan–Meier method and compared using the log-rank test and the Cox proportional hazards model. All the tests were two-sided, and a p-value <0.05 was considered indicative of statistical significance.

Table I. *Patient characteristics*.

	Patient total (n=20)
Age, Median (Range), years	72 (54-85)
Gender, n (%)	
Male	12 (60%)
Female	8 (40%)
ECOG PS, n (%)	
0	13 (65%)
1	7 (35%)
Initial stage	
II/III/IV	9 (45%)/8 (40%)/3 (15%)
Number of metastasis, n (%)	13 (65%)
1/2/3	13/5/2 (65%/25%/10%)
Previous chemotherapy, n (%)	12 (60%)
Metastatic location, n (%)	
Lung	7 (35%)
Liver	11 (55%)
Lymph node	2 (10%)
MTD, Median (Range), mm	20 (7-52)

Results

Twenty samples from 20 patients with oligometastatic CRC were analyzed in this study. The patients' characteristics are shown in Table I. The median age was 72 years (range=54-85 years). Twelve patients (60%) were male, and none had a performance status of ≥2. The metastatic lesions affected the lung (35%), liver (55%), and distant lymph nodes (10%). Thirteen patients (65%) had a single metastasis. Fifteen patients (75%) underwent PBT (relative biological effectiveness: 60-80 Gy/10-33 fractions) and 5 were (25%) treated with SBRT (35-48 Gy/4-5 fractions).

The median amount of cfDNA per sample was 42.1 ng (range=25.3-100.8 ng). The following mutations were detected in the cfDNA from 16 samples (80%): *TP53* (9/20: 45%), *APC* (8/20: 40%), *KRAS* (3/20: 15%), *PIK3CA* (3/20: 15%), *NF1* (1/20: 5%), *BRCA1* (1/20: 5%), *ERBB2* (1/20: 5%), *FBXW7* (1/20: 5%), *KIT* (2/20: 10%), and *HRAS* (2/20: 10%; Figure 1). The maximum allele frequency (MaxAF) was 0.6-7.8% (median 1.5%). Clonal and multi-clonal mutations were confirmed in 11 and five cases, respectively.

The surviving patients were followed for a median of 18 months (range=3-71 months). Recurrence was observed in 13 cases, and the median PFS duration was 10 months (95%CI=5 months-NA). The MaxAF, cfDNA detectability, and mutated gene type were not found to be associated with PFS (Table II). Of the 16 patients in whom cfDNA was detected, those with multi-clonal gene mutation tended to have a worse PFS (14 *vs*. 4 months for clonal, HR=3.1 (95%CI=0.8-11.3), p=0.07, Figure 2a).

Regarding the metastatic lesions of the 4 patients with no cfDNA detected, 3 patients (75%) had lung metastasis.

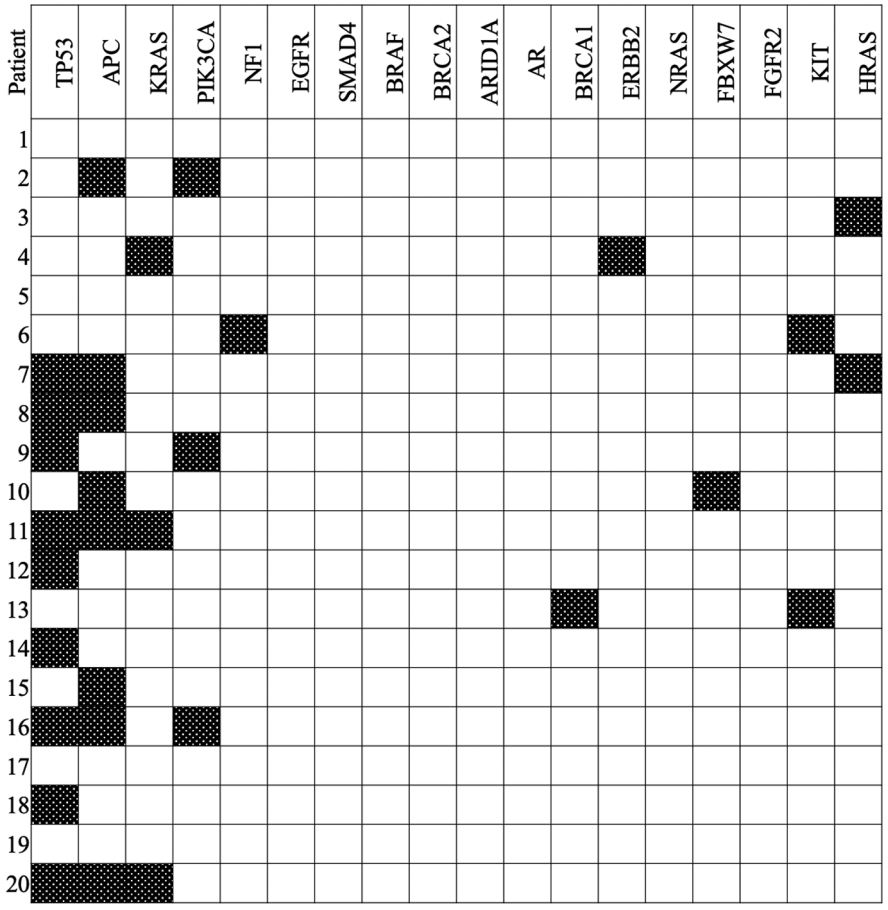

Figure 1. *Mutated genes in 20 plasma samples from patients with oligometastatic colorectal cancer.*

Therefore, additional analyses were carried to determine the impact of detectability of cfDNA on prognosis though the number of patients (7 patients) was limited. As shown in Figure 2b, PFS of the patients with no cfDNA detected was significantly better than that of those with detected cfDNA (p=0.02).

Discussion

In this study, we analyzed the genetic information contained in cfDNA from 20 patients with oligometastatic CRC and detected mutations in at least one of the following genes in 16 patients: *TP53*, *APC*, *KRAS*, *PIK3CA*, *NF1*, *BRCA*, *1ERBB2*, *FBXW*, *7KIT*, and *HRAS*. Additionally, five patients exhibited multi-clonality, and these patients tended to have a relatively worse PFS. This was the first study to focus on the correlation between the cfDNA profile and prognosis in patients with oligometastatic CRC who were treated with ablative radiotherapy.

Table II. *Univariate associations of cell-free DNA (cfDNA) profiles with progression-free survival.*

	HR (95%CI)	*p*-Value
cfDNA detectability	0.9 (0.3-3.5)	0.95
TP53 mutations	0.4 (0.1-1.4)	0.13
APC mutations	0.8 (0.3-2.4)	0.7
KRAS mutations	0.4 (0.1-3.4)	0.42
MaxAF >1%	1.3 (0.4-3.8)	0.68
Clonality	3.1 (0.8-11.3)	0.07

A comparison of the genomic mutations detected in this study with those in other cohorts is shown in Figure 3 (10-12). In our sample, the *HRAS* mutation rate was as high as 10%, whereas the proportion of *KRAS* was relatively low (15%) in the other reports. In the MSK cohort of 1134 patients with metastatic CRC (12), *KRAS* mutation positivity was identified as a poor prognostic factor. In the same cohort, however, the

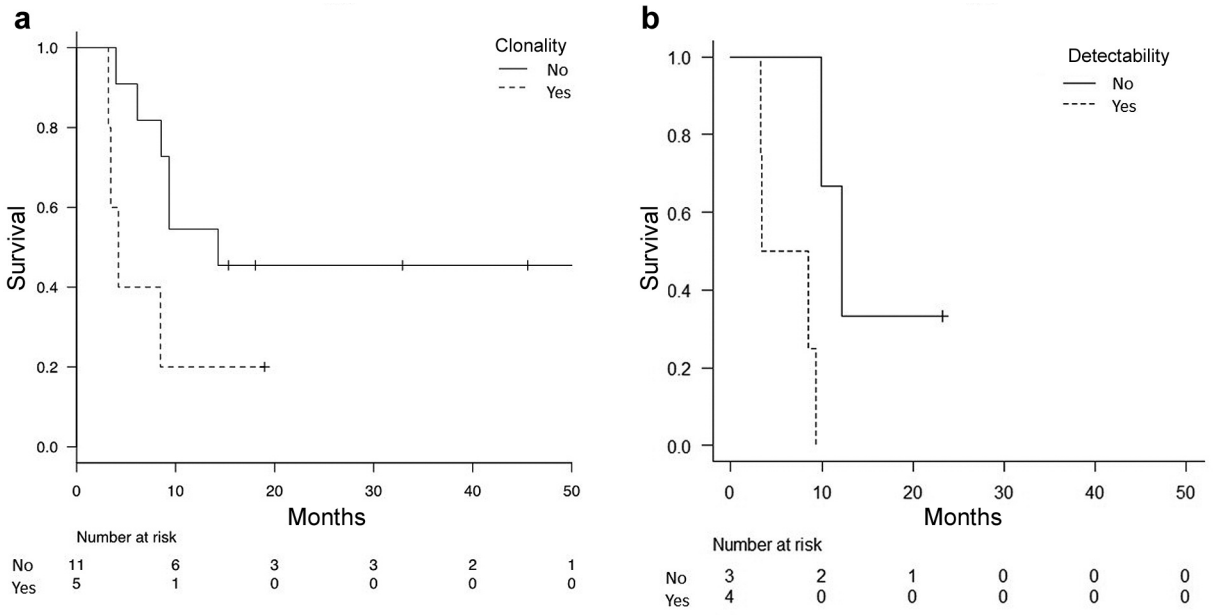

Figure 2. *Progression-free survival curves. (a) Comparison of progression-free survival between patients with clonal versus multi-clonal oligometastatic colorectal cancer. (b) Comparison of progression-free survival depending on detectability of cfDNA among lung oligometastatic colorectal cancer.*

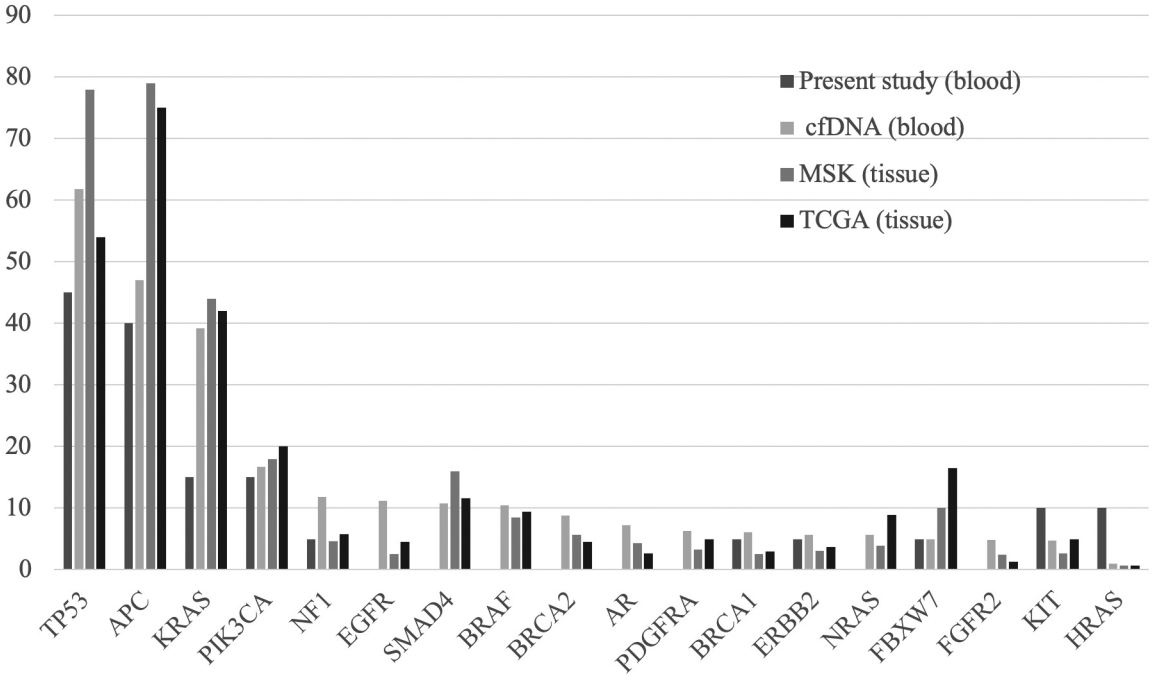

Figure 3. *Comparison of mutation frequencies between the present study and other cohorts.*

very small proportion of *HRAS*-mutation positive patients (0.7%) had a relatively better prognosis when compared to other groups. In addition, the median MaxAF in our study was 1.5%, and this was lower than the rates in other CRC cohorts (10, 13). Elez *et al*. have reported that metastatic CRC patients with a low allele frequency had significantly better overall survival and PFS outcomes after pre-first/second-line chemotherapy (14). The oligometastases included in this study

involved a limited number of metastases in patients whose primary tumors had been controlled by surgical intervention, and it was also called as "oligorecurrence" (15). Studies of various types of primary solid cancer have demonstrated that oligorecurrence is generally associated with a better prognosis among all types of oligometastasis (16). Possibly, patients with oligorecurrence have a better prognosis because of a high frequency of *HRAS* mutations, low frequency of *KRAS* mutations, and low MaxAF, as detected in our analysis.

In this study, we did not observe correlations of the presence or absence of gene mutations with clinical outcomes, including PFS after ablative radiotherapy. However, multi-clonality was associated with a poor PFS in our sample. Several other reports have demonstrated that multiclonal tumors are associated with a worse prognosis than clonal tumors. Andor *et al*. have reported an increased risk of mortality when >2 clones coexisted in the same tumor sample in a pan-cancer analysis (17). Joung *et al*. have described that patients with multiclonal CRC tumors had a worse disease-free survival outcome than those with clonal tumors after surgical resection (18). Our results were partly consistent with those reports, despite differences in the patients' disease backgrounds and treatment modalities. Furthermore, we observed cfDNA detectability was associated with a poor PFS among lung metastasis cases. Patients with advanced colorectal cancer who have only lung metastasis have been reported to have low cfDNA detectability due to the low allele frequency (19, 20). It has been reported that the detectability of cfDNA could be a prognostic factor (21, 22), and it might be a good prognostic marker especially in lung oligometastatic CRC. However, attention must be taken as the detection rate may differ depending on the metastatic organ.

This study has some key limitations. First, the small sample size made it difficult to draw a definite conclusion. To determine the mutation profile derived from cfDNA and investigate its association with prognosis accurately, it will be necessary to evaluate a large cohort with a homogenous background. However, patients with oligometastases from the same primary site are rare, and the accumulation of a large number of patients would be difficult. Therefore, the optimal selection of patients who are suited for oligometastasis-directed ablative therapy remains challenging. Moreover, we lacked reference DNA mutational data from tissue samples. However, a recent report on the genomic landscape of cfDNA in CRC patients demonstrated that the genomic profiles detected in cfDNA were largely consistent with those in tissues (10). We note that cfDNA analysis has other potential advantages, as it can be performed on almost any patient, including those with tumor lesions that are difficult to biopsy or resect. Despite the above limitations, this study provides important information and a future research direction regarding the importance of pre-treatment liquid biopsy in the selection of patients with oligometastatic CRC who would most benefit from ablative therapy.

In conclusion, this is the first study of cfDNA-based genomic profiling in patients with oligometastatic CRC. Our sample had a lower MaxAF and lower *KRAS* mutation-positive frequency and higher *HRAS* mutation-positive frequency than other CRC cohorts. Multi-clonal gene mutation was associated with a poor PFS. Pre-irradiation cfDNA analysis may predict an early recurrence of oligometastatic CRC.

Conflicts of Interest

MN reports personal fees from MSD K. K., personal fees from Astrazeneca, outside the submitted work. All remaining Authors declare no conflicts of interest regarding this article.

Authors' Contributions

MN: made substantial contributions to the conception and design of the study. MN: aided in the collection of data, statistical analysis, and drafting of the manuscript. MO, HH, and AM: assisted the patients data acquisition. SK, MS, and AS: performed analysis of cfDNA. SK and TA: helped to draft the manuscript. All Authors confirm that they have read and approved the final version.

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Availability of data and materials

All the raw sequencing data have been registered in DDBJ under the accession number JGAS00000000196.

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