Clinicopathological and mutational differences between tumors with multiple metastases and single lung metastasis in colorectal cancer

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Abstract. Cancer metastasis, particularly multiple metastatic cancer, is a significant event that affects patient prognosis. However, single metastasis can be treated by partial resection, although the clinicopathological and molecular profile of single lung metastasis has not been thoroughly elucidated. The present study examined tumor heterogeneity by comparing the mutation status between primary colorectal cancer (CRC) and corresponding metastatic lesions to identify prognostic factors associated with single lung metastasis and multiple metastases. The present study enrolled 31 cases of CRC; 20 cases with multiple metastases and 11 cases with single lung metastasis. Clinicopathologically, all cases with multiple metastases were tubular adenocarcinoma, and 3/11 cases with single metastasis were mucinous adenocarcinoma originating from the left side, the remaining 8 cases were tubular adenocarcinoma from the left side. CRC cases with multiple metastases exhibited more frequent vascular invasion, but not lymphatic invasion, than those with single lung metastasis. Furthermore, CRC with multiple metastases was associated with strong tumor budding (P=0.04). Patients with CRC with multiple metastases had lower recurrence-free survival rates compared with those with single lung metastasis (P=0.02). However, there was no significant difference between these two groups in terms of overall survival rates. A next-generation sequencing cancer hotspot panel was used to analyze a heterochronous multiple metastases case, including brain metastasis. Sanger sequencing, immunohistochemistry and microsatellite instability were examined for all 31 cases to reveal the molecular features. *KRAS* and *TP53* mutation signatures were largely preserved throughout the metastatic events. *TP53*/*APC* mutations and overexpression of p53 appeared to be associated with the presence of lymphovascular invasion and strong tumor budding, respectively, although these differences were not statistically significant. Early relapses in patients with CRC could be a sign for eventual multiple metastases, although these may not affect the overall survival of patients with CRC. Considerable mutational changes were seemingly rare during metastatic events in patients with CRC.

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Abbreviations: *APC*, adenomatous polyposis coli; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *CRAF*, Raf-1 proto-Oncogene, serine/threonine kinase; *CTNNB1*, catenin β-1; *GNAS*, guanine nucleotide binding protein, α stimulating complex locus; *KRAS*, KRAS proto-oncogene, GTPase; NGS, next-generation sequencing; *NRAS*, NRAS proto-oncogene, GTPase; PDC, poorly differentiated cluster; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; TB, tumor budding; *TOPO2A*, DNA topoisomerase II α; *TP53*, tumor protein p53; *TERT*, telomere reverse transcriptase

Key words: colorectal cancer, lung metastasis, mutation, *TP53*, *APC*, *KRAS*

Introduction

Metastasis is an important event that defines patients' prognosis during cancer treatment. Cancer cells acquire a variety of phenotypes that allow them to adapt to the distinct tissue microenvironment during the metastatic process. Mutational and epigenetic changes in cancer cells strongly characterize these events. In colorectal cancer (CRC), *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutations occur as oncogenic drivers, which are also expected to play an essential role in these metastatic events.

The liver is the most frequently invaded metastatic organ in CRC, and several studies have demonstrated mutational signatures associated with CRC liver metastasis (1). CRC liver metastasis, even in multiple metastatic cases, can be potentially cured by hepatic resection. Lung metastasis occasionally occurs without liver metastasis; however, mutational signatures associated with single lung metastasis are not well known. Single lung metastasis is also curable by partial resection of the lung. A better understanding of the metastatic potential of primary CRCs would be significantly beneficial in the treatment of CRC patients.

The mutational signature differences between primary and metastatic lesions, especially those associated with single lung metastasis or multiple metastases, has remained unclear. A comparison of the clinicopathological and mutational profiles associated with multiple/single lung metastases in CRC could unravel the fundamental mechanisms underlying tumor metastasis and help to identify early detection biomarkers.

The current study aimed to investigate the clinicopathological and molecular features of tumor heterogeneity by comparing the mutation status between the primary tumor and corresponding metastatic lesions in order to detect factors associated with multiple tumor metastases (which are usually associated with worse prognosis).

Materials and methods

Case selection and histological evaluation. A total of 2,912 cases of CRC were surgically resected at the Juntendo University Hospital (Tokyo, Japan) between 2003 and 2017. We collected data and tissues from 31 CRC cases with lung metastasis (20 with multiple metastases and 11 with single metastasis) from the pathological record. The following clinicopathological factors were evaluated: Gender, age, tumor location, tumor size, histological type, lymphovascular invasion, tumor budding, poor differentiated cluster, perineural invasion, cancer stroma, depth of invasion, lymph node metastasis, distant metastasis, and tumor-node-metastasis (TNM) stage. TNM staging was determined using the 8th UICC TNM staging system of tumors of the colon and rectum (2). The presence of tumor budding (TB) and poorly differentiated cluster (PDC) were evaluated at the invasion front as previously described. TB was counted in the area with the highest density and classified as follows: BD1: 0-4; BD2: 5-9; and BD3: ≥10 (x200 magnification). Furthermore, PDC was classified into three groups: G1, G2, and G3, when they have a maximum number of <5, 5-9, ≥10 PDC, respectively; the counting was done in the highest density area at x200 magnification (3). All patients were followed-up every three months after surgery. The survival periods were determined as survival times after diagnosis. The mean follow-up time was 69.7 months (the range was 18-178 months).

Next-generation sequencing (NGS). A CRC sample with heterochronous multiple metastases, including brain metastasis, was subjected to NGS using the Ion Ampliseq Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Inc.). The details of this NGS analysis are described in our previously published article (4). The patient with the aforementioned sample had experienced liver metastasis on three occasions (in total, six lesions: Three lesions at the first metastasis, two lesions at the second, and one lesion at the third metastasis) followed by lung and brain metastases during the 101 months after initial surgery. Due to poor sample quality, only four recent samples (liver, lung, and two samples from the brain metastasis) from this patient were examined by NGS. Both tumoral and the corresponding non-tumoral DNA were extracted using the QIAamp FFPE tissue kit (Qiagen).

Sanger sequences. Genomic DNA was extracted as previously described (4). Sanger sequencing for *APC* (*Exon 16*), *KRAS* (*Exon 2*), *NRAS* (*Exons 2 and 3*), *GNAS* (*Exon 8*), *BRAF* (*Exon 15*), *CRAF* (*Exons 3, 11, and 14*), *CTNNB1* (*Exon 3*), *PIK3CA* (*Exons 9 and 20*), and *TP53* (*Exons 2,4,5,6,7, and 8*) was performed for all the cases. Furthermore, telomerase reverse transcriptase (*TERT*) promoter mutations were also identified. Primer sequences are described in Table SI. Polymerase chain reaction (PCR) products were cut from the gel and purified by using DNA, RNA, and protein purification kits (MACHEREY-NAGEL). Purified PCR products were sequenced with dideoxynucleotides (BigDye Terminator v3.1; Applied Biosystems; Thermo Fisher Scientific, Inc.), and specific primers were purified using a BigDye X Terminator Purification kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and analyzed with a capillary sequencing machine (3730xl Genetic Analyzer; Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences were then examined by using Sequencing Analysis software version 3.5.1 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Mutations were evaluated by two of the authors (Y.Y. and T.S.) and registered if the mutation peak height reached 20% of the normal peak height. All mutations were verified by sequencing the sense and antisense strands.

Immunohistochemistry. Tissue sections (4 μm) prepared from formalin-fixed and paraffin-embedded tissues were subjected to immunohistochemistry (IHC). Monoclonal antibodies against β-catenin (clone 14, 1:200 dilution; BD Biosciences) and p53 (clone 1801, 1:1 dilution; BioGenex) were used. Antigen retrieval was performed by heating in an autoclave in Tris-EDTA buffer (pH 6.0) for β-catenin and in Tris-EDTA buffer (pH 9.0) for p53. The sections were incubated at 4˚C overnight to react with primary antibodies. Immunohistochemical staining was performed using an Envision kit (Dako) with a substrate-chromogen solution. β-catenin nuclear staining (labeling index) was evaluated in three randomly selected areas by counting the number of positive cells among at least 500 tumor cells at x400 magnification. We judged the tissue as positive for the overexpression of p53 when the number of positive cells >10% of the total cell count (Fig. S1) (4). Slides were evaluated by two independent investigators (Y.Y and T.S.) without prior knowledge of the clinicopathological data. Discrepancies were resolved by reevaluation to reach a consensus.

Microsatellite instability. Microsatellite instability (MSI) analysis was performed using five markers (Bethesda panel: BAT25, BAT26, D5S346, D2S123, and D17S250) (5). Samples with two or more altered markers were classified as MSI-high (MSI-H), samples with one altered marker were classified as MSI-low (MSI-L), and samples without altered markers were classified as microsatellite stable (MSS). We used the primer sets for highly fragmented DNA extracted from the FFPE tissue (6).

Survival analysis and statistical analysis. Correlations between clinicopathological factors and genetic alterations were analyzed by the Fisher's exact test, chi-squared test, and Student's t-test. To elucidate the prognostic impact of each factor, we performed Kaplan-Meier survival analysis and log-rank tests. $P<0.05$ was considered to indicate a

Table I. Clinicopathological differences between colorectal cancer with multiple metastases and single metastasis.

Variable	Multiple (n=20)	Single (n=11)	P-value
Sex, male/female (n)	13/7	8/3	>0.99[a]
Age, years (mean ± SD)	58.6±11.6	60.2±7.4	0.69[b]
Tumor site, right/left/rectum (n)	5/7/8	0/2/9	0.06[a]
Histological type, tublar/mucinous (n)	20/0	8/3	0.04[a]
Ly, 0/1a/1b/1c (n)	9/4/7/0	5/4/2/0	0.50[a]
V, 0/1a/1b/1c (n)	3/8/9/0	7/1/3/0	0.03[a]
Tumor budding, G1/2/3 (n)	14/0/6	10/1/0	0.04[a]
Poorly differentiated cluster, G1/2/3 (n)	4/12/4	1/9/1	0.62[a]
PN, no/yes (n)	15/5	11/0	0.13[a]
Cancer stroma, sci/int/med (n)	2/12/6	1/8/2	0.85[a]
Depth of invasion, T1/T2/T3/T4a (n)	0/3/14/3	0/2/8/1	>0.99[a]
Lymph node metastasis, 0/1/2/3 (n)	6/11/2/1	4/6/1/0	>0.99[a]
TNM I/II/IIIA/IIIB/IV (n)	2/1/6/2/9	1/1/5/1/3	0.86[a]
Size, mm (mean ± SD)	44.4±18.8	37.7±13.3	0.26[b]
Survival period, months (mean ± SD)	72.8±46.8	64.0±11.4	0.77[c]
Recurrence-free period, months (mean ± SD)	6.7±9.0	22.6±22.3	0.02[c]
Mutation rate at the primary site (n)			
KRAS mutated/wild	12/8	7/4	>0.99[a]
TP53 mutated/wild	9/11	3/8	0.45[a]
APC mutated/wild	8/12	3/8	0.70[a]
BRAF mutated/wild	0/20	0/11	>0.99[a]
CRAF mutated/wild	0/20	0/11	>0.99[a]
NRAS mutated/wild	0/20	0/11	>0.99[a]
GNAS mutated/wild	0/20	0/11	>0.99[a]
PIK3CA mutated/wild	0/20	0/11	>0.99[a]
TERT promoter mutated/wild	0/20	0/11	>0.99[a]
Immunohistochemistry (n)			
p53 overexpression positive/negative	11/9	4/7	0.46[a]

[a]Fisher's exact test, [b]student's t-test, [c]log-rank test. TNM stage was analyzed according to the 8th Edition of TNM Classification of Malignant Tumours (UICC). PN, perineural invasion; Ly, lymphatic invasion; V, vascular invasion.

statistically significant difference. These statistical analyses were performed with JMP® ver.12 software (SAS Institute Inc.).

Results

Clinicopathological differences between CRC with multiple metastases and that with single lung metastasis. Clinicopathological differences between CRC with multiple metastases and that with single lung metastasis are summarized in Table I. In this study, the incidence of the single lung metastasis was 0.38% and the cases in which the first metastatic focus was observed in the lung was 0.52% among the multiple metastases group (multiple metastases: 4, single lung metastasis: 11). All the cases with multiple metastases were tubular adenocarcinoma, whereas 3 of 11 cases with single metastasis were mucinous adenocarcinoma. CRCs, which eventually caused multiple metastases, were located evenly from the right of the rectal origin of primary tumors, whereas none of the CRCs with single lung metastasis arose from the right-sided colon, although this difference was not statistically significant (P=0.06). CRC with multiple metastases more frequently showed vascular invasion, but not lymphatic invasion, than those with single lung metastasis. Furthermore, CRC with multiple metastases was associated with strong tumor budding (P=0.04). The presence of PDC did not affect single or multiple metastatic states. CRC patients with multiple metastases presented shorter recurrence-free survival rates compared with those with single lung metastasis with statistical significance (P=0.02). However, there was no significant difference between these two groups regarding overall survival rates. The impact of *KRAS*, *TP53*, and *APC* mutation signatures and IHC of p53 overexpression status on clinicopathological factors were also assessed. *TP53* and *APC* mutations seemed to be associated with the presence of lymphovascular invasion and strong tumor budding, respectively, although these differences were not statistically significant (Tables II and III).

Table II. Association between *TP53* mutation status and clinicopathological factors.

	TP53, n		P-value	
Variable	Wild	Mutated	χ^2	Fisher
Poorly differentiated clusters				
G1/2/3	3/13/3	3/7/2	0.80	>0.99
Budding grade				
G1/2/3	15/1/3	11/1/0	0.34	0.36
PN				
No/yes	16/3	10/2		>0.99
Ly				
No/yes	11/8	4/8		0.17
V				
No/yes	8/11	2/10		0.14
Pathologic type				
tub/muc	18/1	10/2		0.95
Location				
Right/left/rectum	3/5/11	2/4/6	0.90	
pStage				
I/II/IIIA/IIIB/IV	2/1/7/2/6	1/0/4/1/6	0.73	0.93

PN, perineural invasion; Ly, lymphatic invasion; V, vascular invasion; tub, tubular adenocarcinoma; muc, mucinous adenocarcinoma.

Table III. Correlation between *APC* mutaion status and clinicopathological factors.

	APC, n		P-value	
Variable	Wild	Mutated	χ^2	Fisher
Poorly differentiated clusters				
G1/2/3	17/1/2	9/1/1	0.91	>0.99
Budding grade				
G1/2/3	18/0/2	6/1/4	0.06	0.06
PN				
No/yes	17/3	9/2		>0.99
Ly				
No/yes	11/9	4/7		0.27
V				
No/yes	7/13	3/8		0.49
Pathologic type				
tub/muc	17/3	11/0		0.25
Location				
Right/left/rectum	4/4/12	1/5/5	0.30	0.35
pStage				
I/II/IIIA/IIIB/IV	2/1/9/2/6	1/1/2/1/6	0.60	0.51

PN, perineural invasion; Ly, lymphatic invasion; V, vascular invasion; tub, tubular adenocarcinoma; muc, mucinous adenocarcinoma.

The overexpression of p53 tended to be associated with vascular invasion, but this association was not statistically significant (Table IV). No significant association was found with *KRAS* mutation (Table SII).

Table IV. Association between p53 immunohistochemistry and clinicopathological factors.

Variable	p53 overexpression, n		P-value	
	Positive	Negative	χ^2	Fisher
Poorly differentiated clusters				
G1/2/3	4/8/3	2/12/2	0.44	0.51
Budding grade				
G1/2/3	12/0/3	12/1/3	0.51	>0.99
PN				
No/yes	12/3	14/2		0.65
Ly				
No/yes	7/8	8/8		>0.99
V				
No/yes	3/12	7/9		0.25
Pathologic type				
Tub/muc	13/2	15/1		0.60
Location				
Right/left/rectum	3/5/7	2/4/10	0.67	0.69
pStage				
I/II/IIIA/IIIB/IV	1/1/6/1/6	2/1/5/2/6	0.51	>0.99

PN, perineural invasion; Ly, lymphatic invasion; V, vascular invasion; tub, tubular adenocarcinoma; muc, mucinous adenocarcinoma.

KRAS mutation signatures according to metastatic events (Fig. 1). ***KRAS*** mutations were found in 12 out of 20 cases with multiple metastases at the primary sites, and in 7 out of 11 cases with single metastasis at the primary sites. ***KRAS*** mutation signatures were maintained throughout the metastatic events in most cases. In two cases (Case #M19 and #S2), clones different from the primary sites were detected at the metastatic sites, and in one case (Case #M20), clones with ***KRAS*** mutation were first detected at the metastatic site, whereas no ***KRAS*** mutation was detected in the primary tumor. Both metastatic brain lesions in Case #M5 harbored ***KRAS*** mutations similar to that of the primary tumor, and only two out of the other seven metastatic lesions contained ***KRAS*** mutations.

TP53 mutation signatures and p53 immunohistochemistry, and the relationship with metastatic events (Fig. 1). ***TP53*** mutations were found at the primary sites in 9 of 20 cases with multiple metastases, and in 3 of 11 cases with single metastasis at the primary sites. In one patient (Case #M14), a***TP53*** mutation was detected in the latest metastatic lesion, despite the absence of a ***TP53*** mutation at the primary site. In another patient (Case #S7) with single metastasis, a ***TP53*** mutation was detected only in the primary site. In most cases, ***TP53*** mutation signatures were preserved throughout the multiple metastatic events. Regarding a patient with metastatic brain lesions (Case #M5), five out of seven metastatic lesions contained ***TP53*** mutations, in addition to the brain metastatic tumors. The overexpression of p53 was observed in 11 of 20 multiple metastatic cases at the primary site (55.0%), 9 of which had a ***TP53*** mutation. Eight of 9 cases with a mutation in the primary site showed overexpression of p53 in all the metastatic sites; however, ***TP53*** mutations were not preserved in all of the metastatic lesions (Cases #M1, #M5, and #M7). In Case #M5, the overexpression of p53 was not observed in two metastatic lesions of the liver, one of which contained a ***TP53*** mutation. In Case #M14, p53 overexpression was detected only in metastatic lesions. A ***TP53*** mutation was absent in the primary site and detected only in the second metastatic site in the lung. In patients with single metastasis, the overexpression of p53 was observed in 4 of 11 cases (36.3%), 3 of which had a ***TP53*** mutation in the primary tumors. In one of the three cases with single lung metastasis, the overexpression of p53 was also observed at the metastatic site without a mutation (Case #S7). We also observed the overexpression of p53 both at the primary and metastatic sites in three cases without a ***TP53*** mutation (Case #M6, #M11, and #S3). There were no statistically significant differences between multiple metastases and single lung metastasis in terms of mutation ratio and overexpression of p53 (P=0.45 and P=0.46, respectively).

APC mutation signatures according to the metastatic events (Fig. 1). ***APC*** mutations were detected in 8 out of 20 cases with multiple metastases at the primary sites, and in 3 out of 11 cases with single metastasis at the primary sites. All mutations were considered frameshift or nonsense, and the mutation signatures were preserved in most cases. In one case (Case #M11), frameshift and nonsense mutations were preserved from the primary tumor to the three metastatic lesions. In another case (Case #M13), ***APC*** mutation was detected only in one of two metastatic sites without mutation at the primary site. Regarding the case with metastatic brain lesions (Case #M5), six out of seven metastatic lesions contained ***APC*** mutations in addition to the brain metastatic tumors.

Figure 1. *KRAS*, *TP53* and *APC* mutations and p53 immunohistochemical signatures according to metastatic events. These mutations were preserved throughout the course of the metastatic events in most cases, although a few cases acquired novel mutations at the metastatic sites. p53 overexpression was mostly conserved throughout the metastatic process. The different colors indicate the positions of gene mutation and the status of the immunostaining as shown on the right. Li, liver; Lu, lung; P, pancreas; PD, peritoneal dissemination; S, spleen; B, brain; Ap, appendix; LR, local recurrence.

Clinicopathological and molecular differences between CRC with multiple lung metastases and that with single lung metastasis. There were two cases of multiple lung metastases without metastasis to other organs (Case #M10 and #M16). The primary tumors were located in the rectum in both cases, and both tumors harbored a *KRAS* mutation. Furthermore, these two cases tended to show histologically higher PDC grade, BD grade, and vascular invasion compared with those with single lung metastasis. It seemed that the primary tumors that can eventually cause multiple lung metastases have higher metastatic potential compared with single lung metastatic cases. However, there were no significant differences between any clinicopathological and molecular characteristics of multiple lung metastases and those of single lung metastasis, although the study was limited by the small sample size (Table SIII).

Sanger sequencing for other genes. Sanger sequencing was performed for *TERT, CTNNB1, BRAF, CRAF, NRAS, PIK3CA*, and *GNAS*. No hot spot mutations were detected in these genes.

Wnt signal activation in metastatic CRC. Due to the frequency of *APC* (40.0% in multiple metastatic cases, 27.3% in single lung metastatic cases) and *CTNNB1* (0%) mutations, the mutations in these series of cases seemed to be relatively rare

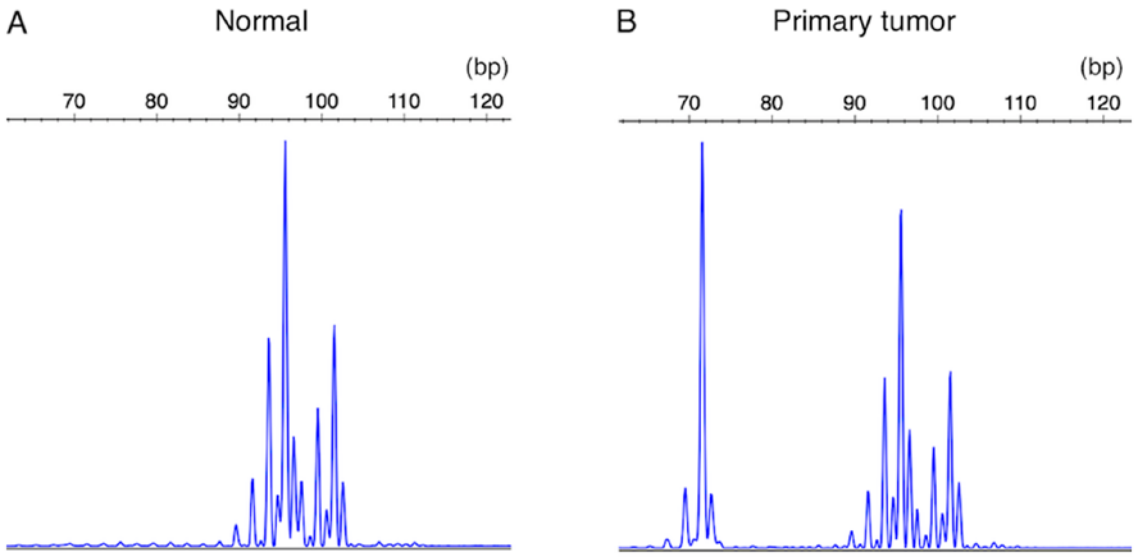

Figure 2. Microsatellite instability analysis. Microsatellite instability-low was identified in one of the multiple metastatic cases (case #M7). Compared with the (A) normal sample, smaller size of the PCR product was observed in (B) a tumor sample.

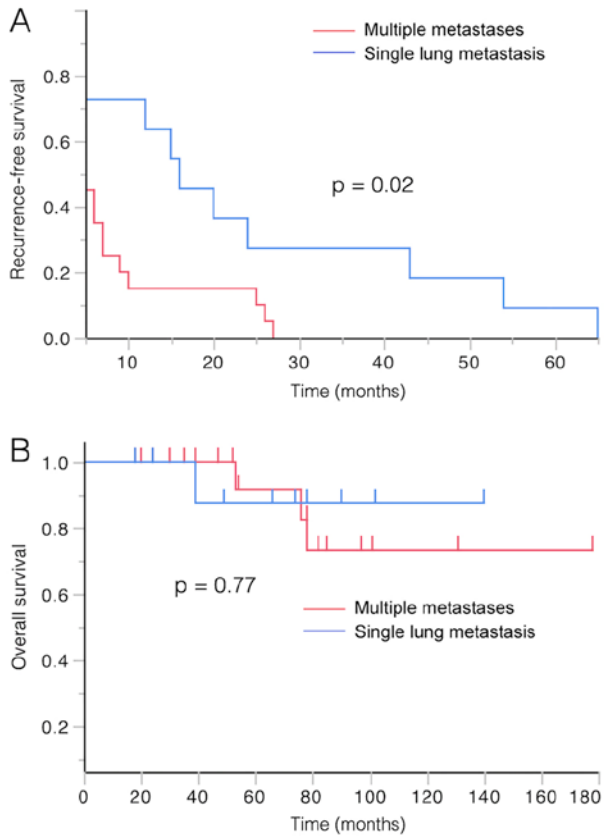

Figure 3. Recurrence-free survival and overall survival rates in patients with colorectal cancer according to the metastatic status. (A) Recurrence-free survival was significantly shorter for patients who eventually experienced multiple metastases than for patients with single metastasis (P=0.02). (B) However, the overall survival rate was slightly worse for patients who experienced eventual multiple metastases, but this was not statistically significant (P=0.77).

compared with reported values (7-9). The status of Wnt signal activation was assessed by β-catenin nuclear staining. Four out of 20 cases of multiple metastases did not show β-catenin nuclear staining, whereas one of them harbored an *APC* mutation. The average β-catenin nuclear labeling index was 34.3% in multiple metastatic cases and 40.7% in single lung metastatic cases (Table SIV).

Status of microsatellite instability in metastatic CRC. The status of MSI was also assessed for the primary tumors. MSI-L was found in only two of the multiple metastatic cases (Case #M7 and #M16) and the remaining tumors were classified as MSS (Fig. 2).

Prognostic impacts of clinicopathological factors and metastatic state. TNM stage at the time of the primary surgery significantly affected the patients' overall survival rate (OSR) and time to the first recurrence (TFR). TFR was significantly shorter for the patients who eventually experienced multiple metastases than for patients with single metastasis (Fig. 3A; P=0.02). However, although OSR was slightly worse for patients who experienced eventual multiple metastases, this finding was not statistically significant (Fig. 3B; P=0.77).

Discussion

It has been reported that lung metastasis occurs in 0.67-15.8% of CRC (10,11). In this study, the incidence of single lung metastasis was 0.38%, and the cases in which the first metastatic focus was observed in the lung was 0.52% of the multiple metastases group. The incidence rate in our study seems to be lower than previously reported values. However, the previous studies probably included 'suspected cases' examined only by computed tomography (CT) or plain radiography, and the present study only analyzed cases that were surgically resected and histologically proven as metastasis. These differences might have influenced the difference in the incidence. However, our findings suggest that the first metastatic event can be observed in the lung in approximately 0.9% of CRC cases.

In this study, all CRC cases of right-sided origin belonged to the multiple metastatic group, which is consistent with the

finding that right-sided primary CRCs show worse prognosis than left-sided primary tumors (7,8,12). In contrast, it was unexpected that the single lung metastatic group contained three cases of mucinous carcinoma, and none were found in the multiple metastatic group. Mucinous carcinoma, except for those with MSI-H, tends to show worse prognosis than conventional adenocarcinoma (13-16). We examined MSI status in the cases, but MSI-H was not found in any of the cases. Therefore, the reason for the single metastasis of the aggressive mucinous carcinoma to the lung, but not to other organs, is unclear.

We included PDC and TB as pathological factors and evaluated if these factors are associated with metastatic status, as a recent study demonstrated that a grading system using PDC and TB in neoplastic cells is a strong predictor of nodal metastases and adverse outcome in colon cancer (17). Strong TB was frequently observed in the group with multiple metastases and this finding was statistically significant; however, PDC did not affect the metastatic state. This finding provides us with a possible therapeutic strategy for multiple metastases in CRC patients with strong TB. Furthermore, the impact of *KRAS, TP53*, and *APC* mutation signatures on clinicopathological factors was also assessed. To date, the impact of the mutation signatures on PDC and TB has not been reported. This study revealed that *TP53* and *APC* mutations were able to indicate the presence of lymphovascular invasion; however, none of the genetic alterations was related to PDC and TB.

A recent study demonstrated that right-sided primary microsatellite stable CRC is associated with shorter survival rate and increased mutation ratio (18). In this study, CRCs with multiple metastases were almost evenly distributed in the right/left/rectal regions, whereas none of the CRCs with single lung metastasis arose from the right-sided colon. The occurrence of single lung metastasis was associated with the location of the primary tumors. However, the location of the primary tumor did not affect the eventual multiple metastases in this series of CRCs, which almost completely comprised MSS with only two cases of MSI-L.

Clonal evolution plays an essential role in the metastatic process of CRC. Regarding this point, different clones of *KRAS* mutations were identified at the metastatic lung sites in two cases (Case #M19 and #S2). However, a drastic change in genetic alterations could not be detected in any of the genes analyzed in this study. This is consistent with previous findings indicating that the rate of epigenetic change has been estimated to be orders of magnitude higher than that of genetic alterations and is considered the primary determinant of clonal evolution (19-21). Mutated clones were observed in the metastatic lesion, one case each from multiple metastatic and single lung metastatic tumors, despite the presence of the wild-type alleles in the primary tumor. However, *KRAS, TP53, and APC* mutation signatures seemed to be preserved throughout the metastatic events in cases with detected mutations. Therefore, the analysis of cell-free DNA or DNA derived from circulating tumor cells to detect the mutations in the primary tumor may be helpful for the early detection of CRC metastasis.

In this series, *APC* mutations were detected in 11 cases (8 in the primary tumors from multiple metastatic cases and 3 in the primary tumor of the single metastatic case), and *CTNNB1* mutations including in-frame deletions were not found in any of the cases. The frequency of *APC* mutations has been shown in up to 90% of colorectal adenocarcinomas, and the frequency of *CTNNB1* mutations was reported to be less than 10% in colorectal adenocarcinoma (7,18,22). However, the prognostic value has not been clearly shown. In contrast, it has been shown that CRC with wild type *APC* has a worse prognosis than those with *APC* mutations (23). Although the frequency of *APC* mutations seemed lower than the reported value, but this is probably due to the fact that this study involved only metastatic tumors.

In addition, we came across a case (Case #M5) with brain metastasis in CRC. Brain metastasis in patients with CRC is reported to be rare, and little is known regarding the mutations involved in this process. A previous study analyzed molecular profiling in 30 cases of metastatic brain samples out of 2010 samples with metastatic CRCs and demonstrated that brain metastasis showed the highest *KRAS* mutation rate (24). In this study, both lesions of brain metastasis contained *KRAS* mutations similar to that detected in the primary tumor, whereas only two out of seven metastatic tumors from the same patient contained this type of mutation. Our findings are consistent with previous findings and also provide evidence that anti-epidermal growth factor receptor therapy is not effective for the treatment of metastatic brain tumors in CRC.

Regarding liver metastasis, 12 out of 20 cases in multiple metastatic tumors harbored *KRAS* mutations in the primary tumor, and 8 of these 12 patients developed liver metastasis. Seven out of 14 metastatic liver samples from the eight patients did not harbor *KRAS* mutations. Liver metastasis is associated with *TOPO2A* gene amplification but not with a high frequency of *KRAS* mutation, although *TOPO2A* gene amplification was not examined in our series (24). A previous study reported that tumors with *KRAS* mutations were more likely to develop lung metastasis. However, the overall survival did not differ according to the *KRAS* status (25). In this study, 12 out of 20 (60.0%) cases with multiple metastases and 7 out of 11 (63.6%) cases with single lung metastasis harbored a *KRAS* mutation at the primary sites.

Furthermore, there was no significant difference between the two groups with regards to overall survival. Thus, *KRAS* mutation status did not affect single/multiple lung metastases in this study. Moreover, in this study, *BRAF* mutations were not detected in many cases, which is consistent with previous findings (24).

With respect to treatment, the general condition of the patients allowed the resection of the metastatic lesions on several occasions in most of the patients with multiple metastases. It is reported that the resection of liver and lung metastases provides good long-term survival (26). All patients who succumbed from the disease (multiple metastasis: 3, single lung metastasis: 1) could not continue with the postoperative adjuvant chemotherapy or undergo subsequent surgery due to poor performance status and the side effects of the drugs.

Finally, CRC is known, in general, to initially spread to the liver, and then to the lung and the brain. It was reported that synchronous liver and pulmonary metastases occur in 45 to 70% of patients with CRC (9). Besides, lung metastasectomy in patients with previously resected liver metastases showed a significantly better five-year survival (27). Closer observation of the liver and lung metastases is needed to improve the prognosis of patients. The rationales for comparing single lung metastasis and multiple lung metastases in CRC in the current study are as

follows: (1) clinicians need to carefully follow-up the patients who experienced early relapse, as they have a higher risk of multiple metastasis in the near future. (2) lung metastasis from CRC is usually encountered after liver metastasis. In the case of single lung metastasis after CRC, the possibility of primary pulmonary adenocarcinoma with enteric differentiation needs to be ruled out (28,29). Frequently conserved mutations in *TP53*, *APC*, and *KRAS,* together with p53 IHC findings, could help to distinguish metastasis from primary pulmonary adenocarcinoma with enteric differentiation.

In conclusion, early relapses in CRC patients could be a sign of eventual multiple metastases, although this may not affect the overall survival of CRC patients. Drastic mutational changes seem rare during metastatic events in CRC patients.

A few limitations can be considered in this study. First, the numbers of the cases were too small to draw definitive conclusions. The sample number should be the same in each group. However, based on available pathological records, we found only 31 CRC cases with lung metastasis (20 with multiple metastases and 11 with single metastasis) from amongst 2,912 cases of CRC. Therefore, it is not possible to increase the number of cases. More sample accumulation is necessary to find more persuasive correlations or differences. Second, we verified mutation findings only in *TP53* but not in *APC* and *KRAS*. We employed p53 IHC to verify the mutation findings, because it is well known that p53 IHC antibody is able to detect mutated p53 as overexpression. However, there are no commercially available IHC antibodies that can efficiently detect mutated APC and KRAS, making it difficult to confirm the mutation findings.

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TS, TH and TY planned this study and diagnosed the surgical specimens. YY, YA, NY and ST performed the experiments and analyzed the data. YY and TS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Juntendo University, Tokyo, Japan (approval no. 17-214), and all patients provided written informed consent prior to enrollment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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