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Inter- and intra-tumor profiling of multi-regional colon cancer and metastasis

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ABSTRACT

Intra- and inter-tumor heterogeneity may hinder personalized molecular-target treatment that depends on the somatic mutation profiles. We performed mutation profiling of formalin-fixed paraffin embedded tumors of multi-regional colon cancer and characterized the consequences of intra- and inter-tumor heterogeneity and metastasis using targeted re-sequencing.

We performed targeted re-sequencing on multiple spatially separated samples obtained from multi-regional primary colon carcinoma and associated metastatic sites in two patients using next-generation sequencing. In Patient 1 with four primary tumors (P1-1, P1-2, P1-3, and P1-4) and one liver metastasis (H1), mutually exclusive pattern of mutations was observed in four primary tumors. Mutations in primary tumors were identified in three regions; *KARS* (*G13D*) and *APC* (*R876**) in P1-2, *TP53* (*A161S*) in P1-3, and *KRAS* (*G12D*), *PIK3CA* (*Q546R*), and *ERBB4* (*T272A*) in P1-4. Similar combinatorial mutations were observed between P1-4 and H1. The *ERBB4* (*T272A*) mutation observed in P1-4, however, disappeared in H1. In Patient 2 with two primary tumors (P2-1 and P2-2) and one liver metastasis (H2), mutually exclusive pattern of mutations were observed in two primary tumors. We identified mutations; *KRAS* (*G12V*), *SMAD4* (*N129K*, *R445**, and *G508D*), *TP53* (*R175H*), and *FGFR3* (*R805W*) in P2-1, and *NRAS* (*Q61K*) and *FBXW7* (*R425C*) in P2-2. Similar combinatorial mutations were observed between P2-1 and H2. The *SMAD4* (*N129K* and *G508D*) mutations observed in P2-1, however, were nor detected in H2. These results suggested that different clones existed in primary tumors and metastatic tumor in Patient 1 and 2 likely originated from P1-4 and P2-1, respectively.

In conclusion, we detected the muti-clonalities between intra- and inter-tumors based on mutational profiling in multi-regional colon cancer using next-generation sequencing. Primary region from which metastasis originated could be speculated by mutation profile. Characterization of inter- and inter-tumor heterogeneity can lead to underestimation of the tumor genomics landscape and treatment strategy of personal medicine.

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1. Introduction

Colorectal cancer is the second most prevalence cancer among males (9%) and the third among females (8%) [1–5]. Approximately 20% of patient with colorectal cancer have distant metastasis at the time of diagnosis [3]. The earlier the T stage is the lower the possibility of distant metastasis. One study reported that 1.9% of T1 or T2 colon cancer patients presented distant metastasis preoperatively, and 3.37% presented after a median of 40.7 months during the follow-up period [6]. Despite some recent progress in the treatment including molecular target therapy, patients affected by metastatic colorectal cancer have a 5-year survival rate of approximately 10% [1–5]. Therefore, further understanding of the molecular biology of this disease is needed.

Several studies have revealed genetic heterogeneity of tumors affecting their malignant phenotype. Frequencies of the somatic mutation of oncogenes and tumor suppressor genes vary among tumors of different tissues, which is also discussed as the inter-tumor and intra-tumor heterogeneity [7,8]. Mutations of suppressor genes such as *APC* and *TP53*, for instance, are common in colorectal cancer. The loss of function of these genes is related to the genetic instability, DNA repair ability, and apoptosis induced by DNA damaging agents [9–11]. Thus the mutations of suppressor genes in tumors influence the sensitivity to cytotoxic agents.

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Furthermore, the oncogenic mutation of *RAS* is predictive marker for treatment with anti-EGFR antibody cetuximab or panitumumab in combination with chemotherapy against colorectal cancer [12–15]. Thus elucidation for the heterogeneity of such mutations is important issue for personalized treatment in colorectal cancer.

Next generation sequencing (NGS) is a widely-used technology for the gene mutation analysis. Since NGS can decide the allele frequency of mutations, it can be useful to investigate the genetic heterogeneity of tumors. However, the NGS analysis has been limited for clinical formalin-fixed paraffin-embedded (FFPE) samples. In this study, we performed a multiple gene mutation analysis for FFPE samples using targeted re-sequencing in order to elucidate the tumor heterogeneity.

2. Materials and methods

2.1. Patients and samples

A total of two patients whose multi-regional primary colon carcinoma and associated metastasis had been surgically resected in Kinki University Hospital between April 2009 and March 2014. The staging was determined according to the TNM classification. This study was approved by the ethics committee of Kinki University Faculty of Medicine (Authorization Number: 25-082). All patients in the study provided written informed consent for the use of resected tissue.

2.2. DNA extraction

The FFPE specimens were subjected to a histological review, and only those containing sufficient tumor cells (at least 75% tumor cells) as determined by hematoxylin and eosin staining were subjected to DNA extraction. DNA was purified with the use of an Allprep DNA/RNA FFPE Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quality and quantity of the DNA were verified using the NanoDrop 2000 device (Thermo Scientific Wilmington, DE) and PicoGreen dsDNA assay kit (Life Technologies, Foster City, CA). The extracted DNA was stored at −80 °C until the analysis.

2.3. DNA sequencing

We used 10 ng of DNA for the multiplex PCR amplification using the Ion AmpliSeq Library Kit and the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) according to the manufacturer's instructions. The genes in the Ion AmpliSeq Cancer Hotspot Panel v2 are listed in Supplementary Table S1. The Ion Xpress Barcode Adapters (Life Technologies) were ligated into the PCR products and purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Purified libraries were pooled and sequenced on an Ion Torrent PGM device (Life Technologies) using the Ion PGM 200 Sequencing Kit v2 and the Ion 318 v2 Chip Kit.

DNA sequencing data were accessed through the Torrent Suite v.3.4.2 software program. Reads were aligned against the hg19 human reference genome, and variants were called using the variant caller v 3.6. Raw variant calls were filtered out using the following annotations: homozygous and heterozygous variants, quality score of <100, depth of coverage <19.

Known SNPs were excluded using the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB) [16].

3. Result

3.1. Patient 1

A 55 years old male had multifocal sigmoid colon cancers, and all tumors were surgically resected as a whole, completely (Fig. 1A). Two tumors (P1-1 and P1-2) had a moderately differentiated histology and pathologically reached subserosa (pT3). The others (P1-3 and P1-4) had a well differentiated histology and reached submucosa (pT1). There were multi lymph node metastases (pN2). Then, the patient received adjuvant chemotherapy (8 courses of capecitabine and oxaliplatin; XELOX). Eight months later, single liver metastasis was detected and the patient received neoajuvant treatment of XELOX plus bevacizumab. The response was stable disease and he received a partial hepatectomy. We obtained FFPE samples of four primary tumors (P1-1, P1-2, P1-3, and P1-4) in sigmoid colon and one region in liver (H1). The clinical course and clinicopathological features are summarized in Fig. 1A and Table 1, respectively.

Targeted re-sequencing was performed using the Ion AmpliSeq cancer hotspot panel v2. Intronic changes and exonic SNPs on HGVD database were excluded in our analysis as described in Materials and Methods. Non-synonymous somatic point mutations that change the protein amino acid sequence are summarized in Table 2. Mutations in primary tumors were identified in three regions; *KARS* (*G13D*) and *APC* (*R876**) in P1-2, *TP53* (*A161S*) in P1-3, and *KRAS* (*G12D*), *PIK3CA* (*Q546R*), and *ERBB4* (*T272A*) in P1-4. Mutations in each region were mutually exclusive. These results suggested the presence of clonal difference (inter-tumor heterogeneity) between the regions. Comparing the mutation profile between primary regions and metastatic region (H1), *KRAS* (*G12D*) and *PIK3CA* (*Q546R*) were shared by P1-4 and H1 but not the others. High allele frequencies of *KRAS* (*G12D*) were detected in P1-4 (42.3%) and H1 (56.9%). No additional mutation was detected in H1. Thus, the mutations shared with P1-4 and H1 were detected at higher frequencies than those shared with the others. It was likely that the clone in metastatic region originated from P1-4. Interestingly, the *ERBB4* (*T272A*) mutation disappeared in H1. These findings suggest that P1-4 has intra-tumor heterogeneity and the clones without the *ERBB4* (*T272A*) mutation created the liver metastasis (Fig. 2A).

3.2. Patient 2

An 84 years old female had cecal and sigmoid colon cancers with a single liver metastasis. She received a subtotal colectomy and subsegmental hepatectomy. Both primary tumors had a moderately differentiated histology and pathologically reached subserosa (pT3). The cecal cancer had a partially mucinous histology and the liver metastasis had a poorly differentiated histology. There was no lymph node metastasis (pN0). We obtained FFPE samples of two tumors in cecal and sigmoid colon (P2-1 and P2-2, respectively) and one region in liver (H2). The clinical course and clinicopathological features are summarized in Fig. 1B and Table 1, respectively. Targeted re-sequencing was performed using the Ion AmpliSeq cancer hotspot panel v2. Intronic changes and exonic SNPs on HGVD database were excluded in our analysis as described in Materials and Methods.

Non-synonymous somatic point mutations that change the protein amino acid sequence are summarized in Table 3. We identified the following mutations; *KRAS* (*G12V*), *SMAD4* (*N129K*, *R445**, and *G508D*), *TP53* (*R175H*), and *FGFR3* (*R805W*) in P2-1, and *NRAS* (*Q61K*) and *FBXW7* (*R425C*) in P2-2. Mutations in each region were mutually exclusive. These results suggested the presence of clonal difference (inter-tumor heterogeneity) between the regions like Patient 1. Comparing the mutation profile between primary regions and metastatic region (H2), *KRAS* (*G12V*), *SMAD4* (*R445**), *TP53* (*R175H*), and *FGFR3* (*R805W*) were shared by P2-1 and H2 but not the others. Based on these mutation profiles, it is likely that the clone in metastatic region (H2) originated from P2-1. Interestingly, *SMAD4* (*N129K*) and *SMAD4* (*G508D*) mutations observed in P2-1

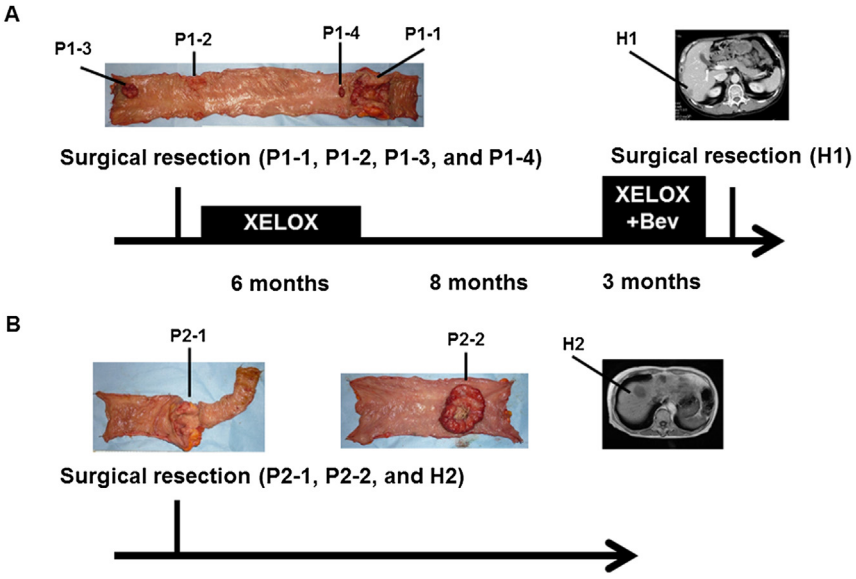

Fig. 1. Treatment timelines for the two patients. A) Patient 1 (a 55-year-old man) had multifocal sigmoid colon cancers, and all of which were surgically resected in their entirety (P1-1, P1-2, P1-3, and P1-4). The patient received adjuvant chemotherapy (8 courses of XELOX). Eight months later, a single liver metastasis (H1) was detected, and the patients received neoadjuvant treatment of XELOX plus bevacizumab. Thereafter, he received a partial hepatectomy. B) Patient 2 (an 84-year-old woman) had cecal and sigmoid colon cancers (P2-1 and P2-2, respectively) with a single liver metastasis (H2). She received a subtotal colectomy and subsegmental hepatectomy.

were not detected in M2. These findings suggest that P2-1 has intra-tumor heterogeneity and the clones without the *SMAD4* (*N129K*) and *SMAD4* (*G508D*) mutations created the liver metastasis (Fig. 2B).

4. Discussion

We investigated the mutation profile of multifocal tumors including metastatic regions to elucidate the inter- and intra-tumor heterogeneity. The targeted re-sequencing of FFPE samples worked well with enough read numbers (average; 3021, range; 2360–3710) of each mutation sites. Comparing the mutation profile between the tumors in the patients, diverse mutations profile were observed in each tumors, suggesting that the inter-tumor heterogeneity exists in a patient.

In Patient 1, both P1-1 and P1-2 were moderately differentiated pT3 tumors, whereas the others were well differentiated pT1 tumors. The recurrence rate has been reported to be about 3% after radical resection of pT1 tumors [17], then clinicians often speculate that most advanced P1-1 or P1-2 creates distant metastases. Surprisingly, however, unique mutations of *KRAS* (*G12D*) and *PIK3CA* (*Q546R*) were shared by P1-4 and H1, and we speculated H1 originated from P1-4. In contrast, unique mutation of *ERBB4* (*T272A*) was detected in P1-4 with high variant allele frequency but was not detected in H1. Basically, *KRAS* (*G12D*) and *PIK3CA* (*Q546R*) are active mutations and these increase the malignant phenotype including metastatic potential [18–20]. There exists multi-clones in P1-4 regions and the clones with the *KRAS* or *PIK3CA* mutations have an aggressive phenotype. Patient 1 received XELOX chemotherapy, and not clones with the *ERBB4* mutation but the aggressive clones with *KRAS* or *PIK3CA* mutations might be resistant to chemotherapy, resulting in development of the liver metastasis (Fig. 2A).

Table 1
Clinicopathological features.

Patient		Lesion	Pathology	T	N
Patient 1	P1-1	Sigmoid colon	Moderately differentiated	3	2
(55-year-old man)	P1-2	Sigmoid colon	Moderately differentiated	3	
	P1-3	Sigmoid colon	Well differentiated	1	
	P1-4	Sigmoid colon	Well differentiated	1	
	H1	Liver metastasis	Moderately differentiated	–	
Patient 2	P2-1	Cecum	Moderately differentiated, partially mucinous	3	0
(84-year-old woman)					
	P2-2	Sigmoid colon	Moderately differentiated	3	
	H2	Liver metastasis	Poorly differentiated	–	

In Patient 2, *KRAS* (*G12V*) was detected in P2-1 and H2 and *NRAS* (*Q61K*) was detected in P2-2. The allele frequency of *KRAS* (*G12V*) was equivalent in P2-1 and H2. No mutation of *NRAS* (*Q61K*) was detected in H2. These results could lead the speculation that H2 originated from P2-1. *SMAD4* mutations were accumulated in P2-1 and H2. The prevalence of *SMAD4* mutations in sporadic colorectal cancer was 8.6% reportedly [21]. These *SMAD4* mutations *N129K*, *R445**, and *G508D* are non-synonymous and loss of function [21–23]. In addition, *SMAD4* mutations are reportedly mutated in mucious phenotype of colorectal cancer [21]. Indeed, P2-1 had a mucinous phenotype. The allele frequency of *SMAD4* (*R445**) was detected in P2-1 with low frequency (1.2%) and H2 with high frequency (34.8%). It could be speculated that there exists multi-clones in P2-1 and the clone with *SMAD4* (*R445**) might be enriched in metastatic regions (Fig. 2B). Loss of function of SMAD4 signal, that located in downstream of transforming growth factor beta signal, promotes *KRAS* driven malignant transforming and metastatisis [24,25]. From these findings, the *KRAS* and *SMAD4* mutations were shared in H2 and this aggressive feature might be related to metastasis.

Anti-EGFR antibodies in combined with cytotoxic agents were one of the standard treatments of patients with colorectal cancer. Mutation status of *RAS* gene is a predictive biomarker for the anti-EGFR antibodies [12–15]. In Patient 1, the *KRAS* (*G12D*) mutation was detected in P1-4 and H1 and the *KRAS* (*G13D*) mutation was detected in P1-2. In Patient 2, the *KRAS* (*G12V*) mutation was detected in P2-1 and H2 and the *NRAS* (*Q61K*) mutation was detected in P2-2. Patients with *KRAS* (*G12D/V*, *G13D*), detected in P1-4, H1, P2-1, and H2, is considered to be resistant to treatment with anti-EGFR antibodies. Patients with *NRAS* (*Q61K*), detected in P2-2, are also considered to be less sensitive to anti-EGFR

Table 2
Non-synonymous mutations in Patient 1.

Chromosome	Position	Gene	Variant effect	Amino acid change	Variant frequency (%)				
					P1-1	P1-2	P1-3	P1-4	H1
Chr2	212587187	*ERBB4*	Missense	T272A	0	0	0	14.8	0
Chr3	178936095	*PIK3CA*	Missense	Q546R	0	0	0	8	27.2
Chr5	112173917	*APC*	Nonsense	R876*	0	27.7	0	0	0
Chr12	25398281	*KRAS*	Missense	G13D	0	5.9	0	0	0
Chr12	25398284	*KRAS*	Missense	G12D	0	0	0	42.3	56.9
Chr17	7578449	*TP53*	Missense	A161S	0	0	25.4	0	0

Chr, chromosome.

antibodies [26,27]. These distinct *RAS* statuses between the tumor regions suggest that the tumor heterogeneity exists in a patient, making it difficult to decide whether anti-EGFR antibodies should be used. In addition, the heterogeneity of *RAS* status raises an additional issue; how many samples in a patients should be tested for *RAS* mutation status prior to the use of anti-EGFR antibody? Repeat biopsies are difficult, invasive and may be confounded by intra-tumor heterogeneity as was seen in our present study [7,8] Recent studies have shown that genomic alterations in solid cancers can be characterized by massively parallel sequencing of circulating cell-free tumor DNA released from cancer cells into plasma, representing a noninvasive liquid biopsy [28,29]. Therefore, such a noninvasive liquid biopsy, whose results might not be influenced by the heterogeneity, can be useful to determine the chemotherapy regimen including molecular targeted agent.

Comparing the primary region and metastatic region, we failed to detect the additional mutations in metastatic regions compared with primary regions in this sample set. In this study, the number of target genes was limited because targeted re-sequencing of Ion Cancer Panel was performed for FFPE samples. The more detailed analysis using exome sequencing or whole RNA sequencing of the frozen samples increases the possibility to find out the novel metastatic related genes.

Table 3
Non-synonymous mutations in Patient 2.

Chromosome	Position	Gene	Variant effect	Amino acid change	Variant frequency (%)		
					P2-1	P2-2	H2
Chr1	11525630	*NRAS*	Missense	Q61K	0	33.4	0
Chr4	1808981	*FGFR3*	Missense	R805W	37.2	0	23.7
Chr4	153247289	*FBXW7*	Missense	R425C	0	33.1	0
Chr12	25398284	*KRAS*	Missense	G12V	33.7	0	29.1
Chr17	7578406	*TP53*	Missense	R175H	54.3	0	34.3
Chr18	48575193	*SMAD4*	Missense	N129K	29.9	0	0
Chr18	48603032	*SMAD4*	Nonsense	R445*	1.2	0	34.8
Chr18	48604701	*SMAD4*	Missense	G508D	9.5	0	0

Chr, chromosome.

In conclusion, targeted re-sequencing of multi-regional colon cancer with metastasis using FFPE samples provided the intra-tumor and inter-tumor heterogeneity in colon cancer based on mutation profile. Metastasis occurred in a clone among the heterogenous tumors. Inter- and intra-tumor heterogeneity might have influence on the effectiveness of molecular targeted agent, such as anti-EGFR antibody, and in such clinical settings, liquid biopsy might be useful. To confirm these findings, further investigation should be performed.

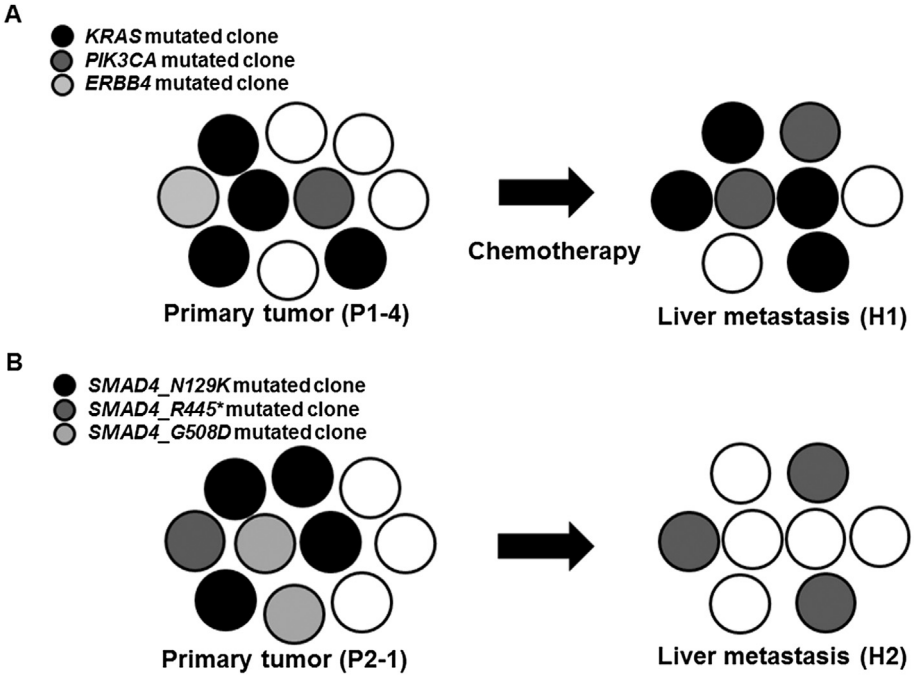

Fig. 2. Schematic representation of intra-tumor heterogeneity in two patients. A) In patient 1, primary tumor (P1-4) contains two or more subclones. The clone without the *ERBB4* (*T272A*) mutation created the liver metastasis. B) In patient 2, primary tumor (P2-1) contains two or more subclones. The clone without the *SMAD4* (*N129K* and *G508D*) mutation created the liver metastasis.

Conflict of interest

All the authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.064.

Transparency document

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