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

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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.

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 profiling. 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 (M1), mutually exclusive pattern of mutations was observed in four primary tumors. Mutations in primary tumors were identified in three regions; KRAS (G13D) and APC (R876*) in P1-2, TP53 (A165S) in P1-3, and KRAS (G12D), PIK3CA (Q546R), and ERBB4 (T272A) in P1-4. Similar combinatorial mutations were observed between P1-4 and M1. In Patient 2 with two primary tumors (P2-1 and P2-2) and one liver metastasis (M2), mutually exclusive pattern of mutations were observed in two primary tumors. We identified mutations; KRAS (G12V), SMAD4 (N129K), NRAS (Q61K) and G508D), TP53 (R175H), and FGFR3 (R805V) in P2-1, and KRAS (G12V), SMAD4 (N129K), NRAS (Q61K) and FBXW7 (R245C) in P2-2. Similar combinatorial mutations were observed between P2-1 and M2. These results suggested that different clones existed in primary tumors and metastatic tumor in Patient 1 and 2 was originated from P1-4 and P2-1, respectively.

In conclusion, we detected the multi-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 intra-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 1.5% 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 between tumors of different tissues. It is also discussed 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. Furthermore, the
oncogenic mutation of KRAS 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). 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). 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 v3.4.2 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. 1). Two tumors (P1-1 and P1-2) had moderately differentiated histology and pathologically reached subserosa (pT3). The others (P1-3 and P1-4) had well differentiated histology and reached submucosa (pT1). There were multi lymph node metastases (pN2). Then, the patients received adjuvant chemotherapy (8 courses of capecitabine and oxaliplatin; XELOX). Eight months later, single liver metastasis was detected and the patients received neoadjuvant treatment of XELOX plus bevacizumab. The response was stable disease and he received 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 (M1). The clinical course and clinicopathological features were summarized in Fig. 1 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 were summarized in Table 2. Mutations in primary tumors were identified in three regions; KRAS (G13D) and APC (R87V) 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 (M1), KRAS (G12D) and PIK3CA (Q546R) were shared by P1-4 and M1 but not the others. High allele frequencies of KRAS (G12D) were detected in P1-4 (42.3%) and M1 (56.9%). No additional mutation was detected in M1. Thus, the mutations shared with P1-4 and M1 were detected at high frequencies than those shared with the others. It was likely that the clone in metastatic region were originated from P1-4. Interestingly, the ERBB4 (T272A) mutation disappeared in M1. These findings suggest that P1-4 has intra-tumor heterogeneity and the clone without the ERBB4 (T272A) mutation creates 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 subtotal colectomy and segmental hepatectomy. Both primary tumors had moderately differentiated histology and pathologically reached subserosa (pT3). The cecal cancer had partially mucinous histology and the liver metastasis had poorly differentiated histology. There were 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 (M2). The clinical course and clinicopathological features were summarized in Fig. 1 and Table 1, respectively. 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 were summarized in Table 3. We identified mutations; KRAS (G12V), SMAD4 (N129K, R445*), and G508D, TP53 (R175H), and FGFR3 (R850W) 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 (M2), KRAS (G12V), SMAD4 (R445*), TP53 (R175H), and FGFR3 (R850W) were shared by P2-1 and M2 but not the others. Based on these mutation profiles, it is likely that the clone in metastatic region (M2) were originated from P2-1. Interestingly, SMAD4 (N129K) and SMAD4 (G508D) mutations observed in P2-1 were not detected in M2. These findings suggest that P2-1
has intra-tumor heterogeneity and the clone with the non-sense SMAD4 (R445*) mutation creates 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 patients.

In Patient 1, both P1-1 and P1-2 had moderately differentiated pT3 tumors, whereas the others had 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 M1, and we speculated M1 was originated from P1-4. In contrast, unique mutation of ERBB4 (T722A) was detected in P1-4 with high variant allele frequency but not detected in M1. 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 and PIK3CA mutations have 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).

In Patient 2, KRAS (G12V) was detected in P2-1 and M2 and NRAS (Q61K) was detected in P2-2. The allele frequency of KRAS (G12V) was equivalent in P2-1 and M2. No mutation of NRAS (Q61K) was detected in M2. These results could lead the speculation that M1 is originated from P2-1. SMAD4 mutations were accumulated in P2-1 and M2. 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 mucinous phenotype of colorectal cancer [21]. Indeed, P2-1 had mucinous phenotype. The allele frequency of SMAD4 (R445*) was detected in P2-1 with low frequency (1.2%) and M1 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 M2 and this 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 KRAS gene is predictive biomarker for the anti-EGFR antibodies [12–15]. In Patient 1, the KRAS (G12D) mutation was detected in P1-4 and M1 and the KRAS (G13D) mutation was detected in P1-2. In Patient 2, the KRAS (G12V) mutation was detected in P2-1 and M2 and the NRAS (Q61K) mutation was detected in P2-2. Patients with KRAS (G12D/V, G13D), detected in P1-4, M1, P2-1, and M2, 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 antibodies [26,27]. These distinct KRAS status between the tumor regions suggests that the tumor heterogeneity exists in a patient. It may be difficult to decide use of anti-EGFR antibodies for the patient. In addition, the heterogeneity of KRAS status debate an issue; how many samples in a patients should be tested of KRAS mutation status for use of anti-EGFR antibody? Repeat biopsies are difficult.
invasive and may be confounded by intra-tumor heterogeneity as is 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 result 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, targeted re-sequencing of Ion Cancer Panel was performed for FFPE samples because the number of target genes was limited. 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.

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 heterogeneous 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.

**Table 2**

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Chr, chromosome.

**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 creates 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 creates the liver metastasis.

**Table 3**

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Chr, chromosome.
A. Kogita et al. / Biochemical and Biophysical Research Communications xxx (2015) 1 – 5

Q3

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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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