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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 (M1), 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 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*, *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 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 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.

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 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

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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 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. 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 neoajuvant 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; *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 (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 higher 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 subsegmental 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* (*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 (M2), *KRAS* (*G12V*), *SMAD4* (*R445**), *TP53* (*R175H*), and *FGFR3* (*R805W*) 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

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