Next-Generation Sequencing-based Test for Resectable Colorectal Cancer in Real-World Clinical Practice

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Research Article

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Abstract

Purpose:

This study aimed to evaluate the significance of Next-generation sequencing (NGS)-based gene panel testing in resectable colorectal cancers (CRC)s by analyzing real-world data collected prospectively from patients.

Methods:

Patients with CRC who underwent surgery from July 2018 to February 2020 at our institution were included, and correlations between various NGS data and clinicopathological findings were evaluated.

Results:

Overall, 107 patients were included in this study. The tumor stage was I in 28 cases (26.2%), II in 40 cases (37.4%), III in 32 cases (29.9%), and IV in 7 cases (6.5%). Actionable gene alterations were found in 97.2% of the cases. Co-alteration analysis suggested that either TP53- or APC-related alterations were more frequently found in early-stage tumors (stage I). The copy number alteration count was significantly lower in right side colon tumors than in tumors in other locations (P < 0.05). Homologous recombination deficiency (HRD) was more often identified in stage IV tumors than in stage I or II tumors (P < 0.05). Moreover, high HRD status was suggested to be useful for identifying high-risk stage II tumors (P < 0.05).

Conclusion:

In this study, real-world NGS data represented the biological features of CRCs. HRD was identified as a useful result of gene panel testing with novel utility in clinical practice.

Introduction

Malignant tumors are traditionally diagnosed and classified based on the organ of origin and histological type, and treatments are selected according to such classification. However, it has become clear in recent years that malignant tumors are caused by the accumulation of various genetic mutations. Hence, treatment strategies against malignant tumors place more emphasis on targeting such genetic mutations. The concept of "precision medicine," in which genetic mutations in individual malignant tumors are analyzed and individualized treatment targeting those mutations is employed, has also been gaining ground in recent years [1]. In the case of colorectal cancer (CRC), it has been reported that tumors are caused by carcinogenic pathways involving various genetic mutations, such as mutations caused by the adenoma-carcinoma sequence (ACS) [2]. Accordingly, comprehensive next-generation sequencing
(NGS) studies, such as those of The Cancer Genome Atlas (TCGA), have been performed and have revealed that CRC can be classified into some molecular subtypes based on genomic events [3].

NGS-based genomic testing is currently used in clinical settings for the practice of precision medicine. Gene panel tests such as MSK-IMPACT™ (New York, NY, USA) and Foundation One® CDx (Cambridge, MA, USA) have been approved by the U.S. Food and Drug Administration (FDA), and their use is spreading to countries worldwide, including Japan [4]. However, the indications for these gene panel tests are limited to locally advanced or metastatic solid tumors for which standard treatment has been completed or advanced solid tumors for which no standard treatment is available. Therefore, currently, very few patients can benefit from testing. In fact, while actionable gene mutations are identified in 37–86% of solid cancer patients, only 10–20% of them are actually identified as targetable mutations [5–7]. Extending the indications of gene panel testing to early-stage tumors may reveal its utility, but no such studies have been conducted thus far.

In our institute, in-house NGS-based gene panel testing, which analyzes 160 oncogenes, is performed for all resectable solid cancer patients in a clinical trial setting. The novelty of this trial is that the gene panel testing is performed immediately after the primary curative surgery, the timing that is not available for other insurance covered testings thus far. In addition, our in-house gene panel testing has cost effectiveness, which is cheaper than other tests. This trial was expected to explore any advantage of genetic information in the decision of treatment after curative surgery.

Here, we report the CRC results: real-world data collected prospectively from all patients with CRC who underwent primary curative surgery at our hospital, including patients with early-stage cancers. The aim of this study was to investigate any additional information on genetic changes during CRC progression and to explore the significance of gene panel testing at primary surgery, which will lead to further expansion of the testing.

**Materials And Methods**

**Patients**

This study included patients with CRC who underwent curative surgery from July 2018 to February 2020 at Keio University Hospital. The study protocol was approved by the ethics committee of Keio University (approved study number: 20180015). All study participants provided informed consent. This study was performed following all relevant guidelines and regulations. The Union for International Cancer Control (UICC) TNM classification was used for stage classification, and the European Society of Medical Oncology (ESMO) Clinical Practice Guidelines were used for high-risk stage II classification: specifically, lymph nodes <12, poorly differentiated tumor, presence of vascular/lymphatic or perineural invasion, pT4 stage, and clinical presentation with intestinal occlusion or perforation [8].

**Next-generation sequencing**
Tumor tissue was collected from surgical specimens of CRC patients who provided consent to undergo comprehensive genomic testing. The details of the panel have been previously reported [4, 9, 10]. Briefly, genomic DNA was extracted from 10-µm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections of tumor specimens using the Maxwell RSC FFPE Plus DNA Kit (Cat. AS1720, Promega, Madison, USA) according to the manufacturer’s instructions. DNA quality was checked by calculating the DNA integrity number (DIN) using an Agilent 4200 TapeStation (Agilent Technologies, Waldbronn, Germany); all analytes had DIN ≥ 2.0. Libraries were generated from 80 (DIN ≤ 2.5) or 160 (DIN > 2.5) ng of DNA per sample using the Human Comprehensive Cancer Panel, GeneRead DNaseq Panel PCR kit V2, GeneRead DNA Library I Core Kit, and GeneRead DNA Library I Amp Kit (Qiagen, Hilden, Germany), and the library quality was assessed using the Agilent D1000 ScreenTape (Agilent Technologies). Targeted amplicon exome sequencing was performed using a 160 cancer-related gene panel as previously described [4, 9, 10]. The targeted regions of all 160 genes were specifically enriched using oligonucleotide probes. The enriched libraries were sequenced with a paired-end (150 bp × 2) sequencing method using the NextSeq sequencing platform (Illumina, San Diego, CA, USA), resulting in a mean depth of 500. The sequencing data were analyzed using the GenomeJack bioinformatics pipeline (Mitsubishi Space Software Co., Ltd., Tokyo, Japan) (http://genomejack.net/) as previously described [11]. The proportion of tumor cells ranged from 5 to 80% (median 45%). Tumor mutation burden (TMB) was defined as the number of nonsynonymous and synonymous mutations in the target. The estimated copy number (CN) of the tumor cells was calculated by the following formula: estimated CN = (measured CN − 2)/proportion of tumor cells + 2. Homologous recombination deficiency (HRD) was evaluated by determining the “HRD score”. The score was calculated using an algorithm similar to the LOH score in Myriad mychoice® CDx (Salt Lake City, UT, USA). Although the LOH score is calculated by the sum of LOH, telomeric allelic imbalance, and large-scale state transitions, the latter two factors cannot be calculated in target-gene panel sequences due to the limited number of genes. Thus, a unique method of counting copy number alterations (CNAs) has been used to ensure measurement sensitivity. In detail, the score was defined as the percentage of detected breakpoints in the whole genome and differences in the CNA status of adjacent probe genes. CNA status includes 3 categories: loss, neutral and amplification. LOH regions spanning ≥ 90% of a whole chromosome or chromosome arm are considered to be due to non-HRD mechanisms [12]. Thus, chromosomes with fewer than 2 probe genes (#8, #18, #21 and X in this test) were excluded from the calculation of the HRD score. In addition, chromosomes with the same CNA status on a single chromosome were also excluded. In this study, an “actionable” gene alteration was defined as pathogenic variants and copy number alterations (CN > 4 or homozygous deletions (HD) or LOH). The annotated and curated analysis report was discussed at a genome expert conference consisting of medical oncologists, molecular oncologists, pathologists, medical geneticists, clinical laboratory technicians, bioinformaticians, genetic counselors, pharmacists, and nurses.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). The Mann-Whitney U test and chi square test were applied as appropriate. The significance level was set as 0.05. Co-alteration analysis results were plotted using Circos, a Perl language-based tool used
to represent visual data in a circular form [13]. The Circos plot was generated by using ClicO FS Circular Layout Interactive Converter Free Services. The details of usage have been previously reported [14].

Results

Patient characteristics

The characteristics of CRC patients who were included in this analysis are shown in Table 1. Overall, sequencing was performed for 107 CRC cases (94 well-differentiated to moderately differentiated adenocarcinoma (WEL), 2 poorly differentiated adenocarcinoma (POR) cases, and 11 mucinous adenocarcinoma (MUC) cases, and the cohort included 59 (55.1%) males and 48 (44.9%) females. The median age was 70 years old (range: 61-76), and the tumor locations were as follows: right side colon, 42 (39.3%); left side colon, 27 (25.2%); and rectum, 38 (35.5%) cases. The tumor stages were as follows: stage I, 28 (26.2%); stage II, 40 (37.4%); stage III, 32 (29.9%); and stage IV, 7 (6.5%).

<table>
<thead>
<tr>
<th></th>
<th>WEL</th>
<th>POR</th>
<th>MUC</th>
<th>All</th>
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<tbody>
<tr>
<td>n</td>
<td>94</td>
<td>2</td>
<td>11</td>
<td>107</td>
</tr>
<tr>
<td>Age</td>
<td>70 (61-77)</td>
<td>57 (52-62)</td>
<td>69 (63-75)</td>
<td>70 (61-76)</td>
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<tr>
<td>Sex</td>
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<tr>
<td></td>
<td>53 (56.4)</td>
<td>1 (50.0)</td>
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<td>59 (55.1)</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>right side colon (%)</td>
<td>34 (36.2)</td>
<td>2 (100)</td>
<td>6 (54.5)</td>
<td>42 (39.3)</td>
</tr>
<tr>
<td>left side colon (%)</td>
<td>27 (28.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>27 (25.2)</td>
</tr>
<tr>
<td>rectum (%)</td>
<td>33 (35.1)</td>
<td>0 (0)</td>
<td>5 (45.5)</td>
<td>38 (35.2)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
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<tr>
<td>I (%)</td>
<td>28 (29.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>28 (26.2)</td>
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<tr>
<td>II (%)</td>
<td>37 (39.4)</td>
<td>0 (0)</td>
<td>3 (27.3)</td>
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<td>III (%)</td>
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<tr>
<td>IV (%)</td>
<td>4 (4.2)</td>
<td>0 (0)</td>
<td>3 (27.3)</td>
<td>7 (6.5)</td>
</tr>
</tbody>
</table>

Summary of patient clinicopathological characteristics in each histological type.

WEL: well to moderately-differentiated adenocarcinoma; POR: poorly-differentiated adenocarcinoma; MUC: mucinous adenocarcinoma

Actionable gene alterations

Among the 111 patients, actionable gene alterations were identified in a total of 104 samples (97.2%): WEL, 91 (96.8%); POR, 2 (100.0%); and MUC, 11 (100.0%). The frequent actionable gene alterations are summarized in Figure 1a and Table 2. Actionable gene variants in mismatch repair (MMR) genes were identified only in WEL: mutations in MSH2 in 4.3% of cases and mutations in MLH1 in 3.2% of cases.
Among the genes related to HRD, an actionable gene variant in $ATM$ was identified in 4.3% of WEL, 100% of POR, and 9.1% of MUC; an actionable gene variant in $BRCA2$ was identified in 6.4% of WEL and 9.1% of MUC; and an actionable gene variant in $PALB2$ was identified in 5.3% of WEL. Regarding tumor suppressor genes (TSGs), actionable gene variants were identified in various genes. Among the TSGs, $APC$ was the most frequently mutated gene and was mutated in 58.9% of all samples, 59.6% of WEL samples, 0% of POR samples, and 63.6% of MUC samples. A $TP53$ actionable gene variant was identified in 54.2% of all samples, 55.3% of WEL samples, 0% of POR samples, and 54.5% of MUC samples. A $SMAD4$ actionable gene variant was identified in 16.8% of all samples and was very frequent in MUC samples: it was identified in 13.8% of WEL samples, 0% of POR samples, and 45.5% of MUC samples. For oncogenes (OGs), a $KRAS$ actionable gene variant was identified in 35.5% of all sample types: WEL, 34.0%; POR, 0%; and MUC, 54.5%. In addition, a $BRAF$ actionable gene variant was identified in 17.8% of all samples: WEL, 17.0%; POR, 50.0%; and MUC, 18.2%. Figure 1b shows the co-alteration analysis results according to tumor stage. In stage I tumors, the majority of co-alterations were either $TP53$- or $APC$-related, whereas other combinations were identified in advanced tumors.

**Copy number alterations**

A heatmap of the CNAs is shown in Figure 2a. Overall, frequent losses of $SMAD4$ (36.4%), $TP53$ (33.6%), and $FAS$ (24.3%) and amplifications of $ASXL1$ (36.4%), $GNAS$ (33.6%), and $HSPH1$ (33.6%) were identified. From the analysis of CNAs of the 160 cancer-related genes, frequent gains of genes in 13q and 20q were identified in WEL, but these were less frequent in MUC. There was no statistically significant difference in CNA count between histological types (Figure 2b). The median CNA counts were as follows: WEL 16.0 (0-96.0) v.s. POR 12.5 (1.0-24.0) v.s. MUC 21.0 (0-46.0) (p = 0.57). The CNA count for each tumor stage was as follows (Figure 2c): stage I 11.0 (0-70.0) v.s. stage II 17.5 (0-82.0) v.s. stage III 14.0 (0-96.0) v.s. stage IV 30.0 (17.0-77.0) (p = 0.04). There was a statistically significant difference between stages I and IV (p = 0.008) and between stages II and IV (p = 0.02). Moreover, tumor location was associated with CNA count (Figure 2d): right side colon 7.0 (0-77.0) v.s. left side colon 17.0 (0-96.0) v.s. rectum 18.5 (0-55.0) (p = 0.045). There were statistically significant differences between right- and left-side CRC (p = 0.03) and between right-side and rectal CRC (p = 0.04).

**Tumor mutation burden**

Regarding TMB, there was no significant difference in any of the comparisons (Figure 3a-c). Of note, the median TMB was marginally higher in right side CRC: right side CRC 8.1 (0-221.5) v.s. left side CRC 6.7 (2.7-76.5) v.s. rectal CRC 8.1 (1.3-17.5) (p = 0.22). Hypermutation, which was defined as TMB $\geq$ 10, was identified in 29 cases (27.1%) in total. However, the proportion of hypermutation cases was not significantly different in any of the comparisons (data not shown).

**Homologous recombination deficiency**
The HRD score was marginally associated with tumor stage (Figure 4b): stage I 0 (0-4.5) v.s. stage II 0 (0-7.5) v.s. stage III 0 (0-8.3) v.s. stage IV 1.5 (0-9.8) (p = 0.05). There were statistically significant differences between stages I and IV (p = 0.02), between stages II and IV (p = 0.005) and between stages III and IV (p = 0.03). We next attempted to use the HRD score as a recurrence risk predictor in stage II patients (Figure 4c). When an HRD score ≥ 1 was defined as a high HRD score, the HRD score correlated well with the high-risk stage II tumors: low risk 0% v.s. high risk 30% (p = 0.049). On the other hand, the HRD score was not associated with histological type or tumor location (Figure 4a, d): WEL 0 (0-9.8); POR 0 (0-0); MUC 0.8 (0-4.1) (P = 0.16); right side colon 0 (0-9.8); left side colon 0.4 (0-8.3); and rectum 0 (0-4.1) (p = 0.20).

**Discussions And Conclusion**

This study presents real-world NGS sequencing data obtained from samples prospectively collected from CRC patients who were eligible for curative surgery. Although there have been many reports on unresectable advanced or metastatic CRCs, the results of this study, in which all the cases were resectable and over 60% were stage I or II cancer, are important for understanding the potential significance of genetic testing. According to this study, TMB and CNA count are associated with pathological histology and tumor location and represent the biological features of tumors. A combination of altered genes represents tumor progression. In contrast, the HRD score is better associated with tumor stage and represents tumor progression, suggesting its possible utility in clinical practice.

Here, we employed an in-house targeted amplicon exome sequencing-based panel including 160 cancer-related genes that has been validated in several solid tumors, such as ovarian cancer and pancreatic cancer [4, 9, 10]. The detection rate of actionable genes in CRC was higher than 90% with this panel, which is comparable with that with other NGS-based oncogene panels [6, 15, 16]. The major driver genes in CRC, including *APC*, *KRAS*, and *SMAD4*, also had mutation rates comparable to those in other studies [17–19]. The slightly lower mutation rate in *TP53* may be because our study included more early-stage cancers than other studies. Interestingly, co-alteration analysis showed that most of the stage I tumors had alterations with either *TP53* or *APC* co-alterations. On the other hand, more advanced staged tumors (stages III and IV) had various gene combinations. This finding may explain ACS, in which *APC* and *TP53* alterations leading to adenoma formation are the first to manifest, and other gene alterations accumulate during malignant transformation [20–22]. Therefore, our results indicate that stage I tumors are closer to adenomas.

Chromosomal aberrations are present in approximately 85% of CRCs, generally involving losses in 8p, 17p and 18q and gains in 7p, 7q, 8q, 13q and 20q [23]. It has been suggested that these events occur during ACS [24]. In particular, gains of 20q were first found through banding analysis in CRC and have been observed in more than 65% of CRC cases, which suggests that the genes encoded on 20q have a key role in contributing to the phenotype of CRC when overexpressed [25, 26]. In this study, less frequent gains of genes in 13q and 20q were found in MUC than in other CRC types. This finding may suggest that
the developmental pathways of mucinous and non-mucinous tumors are different and that the development of mucinous tumors does not rely on ACS.

In this study, the CNA count was related to tumor location. Consistent with other reports, right side colon tumors had lower CNA counts than tumors in other locations [27]. This finding may suggest that cellular genomic instability is more pronounced in left-side tumors than in right-side tumors. In addition, the CNA count tended to increase as the tumor stage advanced. No clear results were proposed ascertaining the existence of a gradual increase in the CNA count between early, invasive and metastatic CRC [28], but it has been suggested that progression from invasive cancer to metastasis is accompanied by an increase in the CNA count [29, 30]. A high CNA count means that the number of genes with amplification and loss is high, most likely representing genomic instability. Our results suggest that genomic instability increases with cancer progression.

Although immunotherapy has proven to be effective in treating cancers and is being approved for various types of cancer, including CRC, the number of patients who can benefit from it is still limited [31]. TMB is an emerging biomarker of sensitivity to immune checkpoint inhibitors and has been shown to be more significantly associated with the response to PD-1 and PD-L1 blockade immunotherapy than PD-1 or PD-L1 expression [32]. In CRCs, TMB is reported to be higher in right side colon tumors than in left side tumors [33]. Although not statistically significant, this study also showed that the TMB was relatively higher in right side colon tumors. The distribution of TMB and the subset of patients with high TMB have not been well characterized and are issues to be elucidated in the future.

HRD has received much attention, primarily in breast cancer treatment, since an underlying mechanism of breast cancer formation has been largely attributed to the HRD pathway [34]. Indeed, the importance of the breast and ovarian cancer susceptibility proteins BRCA1 and BRCA2 has been well documented [35, 36]. Genomic tests, such as Myriad myChoice® CDx (Myriad Genetics, Inc., Salt Lake City, UT, USA), which detects BRCA1 and BRCA2 mutants, have been approved by the FDA and are used to detect biomarkers for PARP inhibitor treatment [37]. Unfortunately, the relationship between CRC and HRD has not yet been fully studied. A few reports have shown that brain metastases of CRC and locally advanced rectal carcinomas exhibit elevated mutational signatures of HRD [38, 39]. In this study, a higher HRD score was clearly correlated with tumor progression, and moreover, it was suggested to correlate well with the high-risk stage II classification. Considering postoperative chemotherapy, this finding could be utilized for patient and regimen selection. In fact, a high HRD is associated with susceptibility to platinum agents in ovarian cancers [40].

In conclusion, real-world NGS sequencing data from resectable CRC represent significant biological features of cancer progression. Evaluating HRD in each tumor is considered to be useful in clinical practice as a novel readout of gene panel testing.

**Declarations**

**Funding:**
The authors did not receive support from any organization for the submitted work.

**Competing interests:**

The authors have no competing interests to declare that are relevant to the content of this article.

**Ethics approval:**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of Keio University School of Medicine (No.20180015).

**Consent to participate:**

Informed consent was obtained from all individual participants included in the study.

**Author contributions:**

M.O. designed, analyzed data, and wrote the manuscript. R.S. collected tissues for NGS, wrote the manuscript, and supervised the study. K.O., K.S., S.M., and Y.K. collected tissues for NGS and clinicopathological data. K.N., E.A., S.T., T.C., and H.N. performed the NGS genomic testing and analyzed the data. All authors reviewed the manuscript.

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Figures
Figure 1

a) Actionable gene variants identified in resectable CRCs. Oncogenic variants and variants of unknown significance (VUS) are shown. b) Circos plots of 7 major mutated genes in stage I to IV CRCs. TMB: Tumor mutation burden; CNA: copy number alteration; dMMR: deficient of mismatch repair; HRD: homologous recombination deficiency; TSG: tumor suppressor gene; OG: oncogene; WEL: well-
differentiated to moderately differentiated adenocarcinoma; POR: poorly differentiated adenocarcinoma; MUC: mucinous adenocarcinoma

Figure 2

a) Copy number alteration (CNA) identified in resectable CRCs. b) CNA count in each histological type. c) CNA count in each tumor stage. d) CNA count in each tumor location. The horizontal bars represent...
median value. * P<0.05; N.S.: not significant; TMB: Tumor mutation burden; CNA: copy number alteration; dMMR: deficient of mismatch repair; HRD: homologous recombination deficiency; TSG: tumor suppressor gene; OG: oncogene; WEL: well-differentiated to moderately differentiated adenocarcinoma; POR: poorly differentiated adenocarcinoma; MUC: mucinous adenocarcinoma

Figure 3

a) Tumor mutation burden (TMB) in each histological type. b) TMB in each tumor stage. c) TMB in each tumor location. The horizontal bars represent median value. N.S.: not significant; WEL: well-differentiated
to moderately differentiated adenocarcinoma; POR: poorly differentiated adenocarcinoma; MUC: mucinous adenocarcinoma

Figure 4

a) Homologous recombination deficiency (HRD) score in each histological type. b) HRD score in each tumor stage. c) Percentages of high HRD score cases in low- and high-risk stage II cases. d) HRD score in each tumor location. The horizontal bars represent median value. P<0.05; N.S.: not significant.