Intratumor heterogeneity (ITH) stands as one of the main difficulties in the treatment of colorectal cancer (CRC) as it causes the development of resistant clones and leads to heterogeneous drug responses. Here, 12 sets of patient-derived organoids (PDOs) and cell lines (PDCs) isolated from multiple regions of single tumors from 12 patients, capturing ITH by multiregion sampling of individual tumors, are presented. Whole-exome sequencing and RNA sequencing of the 12 sets are performed. The PDOs and PDCs of the 12 sets are also analyzed with a clinically relevant 24-compound library to assess their drug responses. The results reveal unexpectedly widespread subregional heterogeneity among PDOs and PDCs isolated from a single tumor, which is manifested by genetic and transcriptional heterogeneity and strong variance in drug responses, while each PDO still recapitulates the major histologic, genomic, and transcriptomic characteristics of the primary tumor. The data suggest an imminent drawback of single biopsy-originated PDO-based clinical diagnosis in evaluating CRC patient responses. Instead, the results indicate the importance of targeting common somatic driver mutations positioned in the trunk of all tumor subregional clones in parallel with a comprehensive understanding of the molecular ITH of each tumor.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed human malignancy worldwide and represents the third most common cause of tumor-associated mortalities in Korea.[1] Regardless of the clinical accomplishments of early detection and prevention that have brought about a general decrease of CRC incidence,[2] acquired resistance to chemo- and targeted therapeutics remains the main cause of advanced CRC-associated morbidity, with scarcely any remedial options available.[3] Intratumor heterogeneity (ITH) and cancer clonal evolution have received increased attention since ITH acquired throughout cancer progression seemingly contributes to therapeutic resistance and therefore a dismal oncologic outcome.[4] Genetic intratumor heterogeneity has been reported in several types of solid tumors, such as renal,[5] breast,[6] esophageal,[7] lung,[8] ovarian,[9] prostate,[10] and pancreatic.[11]
tumors. Multiregional sequencing of spatiotemporally distinct tumor regions makes it possible to follow the evolutionary trajectories of cancer cells, with common ancestral somatic driver mutations positioned within the whole tumor mass, but also with subregional mutations that are restricted to one of the subregional clones. The parental clone develops all the common mutations inherited to subregional clones and the accumulation of subregional genetic changes eventually develops into ITH, which is thought to be the main cause of poor therapeutic responses.\(^{12}\)

CRC presents a high spatial heterogeneity but its implication in heterogeneous drug responses has not been fully investigated. Although multiregional analysis of CRC has been conducted in many groups,\(^{13}^{14}\) sequencing approaches using primary cancer tissues have substantial limitations in connecting molecular ITH to various drug responses. In this respect, the unique ability of organoid technology to capture ITH by establishing independent subregional clones from multiple regions of a single tumor mass is valuable for simultaneously assessing both genetic heterogeneity and its impact on diverse drug responses.\(^{14}^{15}\) Nevertheless, previous CRC studies using organoid technology have mainly focused on the comparison between drug responses of primary and metastatic tumor-derived organoids.\(^{15}\) A few studies have associated the significance of ITH with drug responses of CRC and showed its implications for precision medicine,\(^{16}\) yet the number of patients analyzed by subclonal or subregional organoids derived from a single tumor mass, as well as the number of drugs in the screening panel, were insufficient to draw generalizable conclusions. Besides, the heterogeneous drug responses within a single patient in perspective of genetic and transcriptomic ITH has been seldom interpreted. To obtain a clearer overview of the impact of ITH on heterogeneous drug responses, we prepared 12 sets of PDOs and PDCs consisting of 3–4 subregional clones isolated from multiple regions of single tumors from 12 patients, which were then subjected to massive genomic, transcriptomic, and pharmacological analyses.

Our result not only showed an unexpectedly pervasive high level of heterogeneity of drug responses caused by various molecular heterogeneities among subregional clones in all 12 patients but also provided clone-by-clone comparisons in perspective of molecular ITH to clarify heterogeneous drug responses, and enabled comprehensive prediction of subregional drug resistance by integrating genetic and transcriptomic heterogeneity. Our study also demonstrates the limitations of single biopsy-based precision medicine screening and highlights the importance of targeting trunc mutations shared by all cancer subregional clones in parallel with comprehensive integration of transcriptomic and proteomic heterogeneity with genomic factors in order to prevent unwanted drug resistance caused by ITH.

2. Results

2.1. Subregional Colorectal Cancer Organoids and Cell Lines from 12 Patients Retained the Histological Features of the Original Tumor

We have established a living biobank of patient-derived colorectal cancer organoids (PDOs) and cell lines (PDCs), which will be deposited at the Korean Cell Line Bank (http://cellbank.snu.ac.kr) to be distributed worldwide. The multifocal regions of the primary tumor were designated as S1–S4 before preprocessing for further culture. We successfully generated 43 subregional PDOs and 23 subregional PDCs (eight of which were derived from PDOs rather than directly) corresponding to 12 different patients. This allowed us to biologically capture the ITH of each cancer by multifocal sampling. Subsequently, we performed genomic and transcriptomic analyses of the set from each patient, as well as drug responses to a panel of 24 selected drugs, in order to understand the impact of molecular ITH on the biological responses to cancer therapeutics (overall study design in Figure 1A).

In this study, we used suboptimal culture medium that completely prevents the propagation of normal colon cells within three passages (more than 100 samples tested) (Figure S1A,B, Supporting Information). Table 1 summarizes the clinicopathological information of all 12 patients enrolled in this study. Fingerprinting analysis indicated that in all cases the multifocal PDOs and PDCs derived from the same patient shared >90% of specific loci and were not cross-contaminated (Table S1A, Supporting Information). We also applied our RNA-sequencing data to authenticate that our derivatives were not mixed before sequencing (Table S1B, Supporting Information). The variant profiles of each patient were distinct and overall shared low variant identity with other patients whereas multiregional lines exhibited significantly high correlation score, which suggested that there was no mixing of the lines before sequencing. In line with previous data,\(^{14}\) PDOs varied in their growth rate and morphology (Figure 1B; Figure S1C–O, Supporting Information), e.g., spheroidal, asymmetric, or loosely aggregated. The matched PDCs grew as monolayers of substrate-adherent cells displaying polygonal and spindle morphology (Figure 1B; Figure S1E–O, Supporting Information). Hematoxylin-eosin (H&E) staining of paraffin sections from the PDOs, as well as the corresponding tumors, exhibited a strong concordance in their histopathological features (Figure 1B; Figure S1E–O, Supporting Information). Cytokeratin 20 (CK20) and caudal type homeobox 2 (CDX2), as well as nuclear β-catenin (CTNNB1) and Ki-67 (MKI67), were compared between PDOs and corresponding tumors. Also, the expression patterns of mismatch repair (MMR) genes such as MutL homolog 1 (MLH1), MutS Homolog 2 (MSH2), MutS Homolog 6 (MSH6), and PMS1 Homolog 2 (PMS2) were compared between PDOs and matched tumors. PDOs retained analogous intensity and expression of these markers (Figure 1C; Figure S2A–J, Supporting Information). Two MSI-H tumors (SNU-4376A and SNU-4398) were deficient in MLH1 and PMS2 expression (Figure 1C; Figure S2D, Supporting Information). Overall, these data validated that the histological features and expression patterns of specific CRC markers are well conserved in the PDOs. We further inspected biological signs of ITH among each set of established PDOs. Even though derived from the same tumor mass, SNU-4849S4-TO, one of the subregional clones derived from patient SNU-4849, exhibited a much slower growth rate compared to the other subregional clones from the same origin. There was also intramorphological heterogeneity in the subregional clone, SNU-4374S4-TO. One subregional clone had a spheroidal and asymmetric shape, whereas another subregional clone retained a thin-walled cystic structure with a lumen, as confirmed by H&E staining as well as immunofluorescence imaging (Figure S1D,G, Supporting Information). Taken together, these data validated that we have successfully established 12 sets of subregional PDO as...
Figure 1. Histopathological characterization of patient-derived organoids and cell lines. See also Figures S1 and S2 in the Supporting Information. A) Overall study design. We successfully generated 43 PDOs and 23 PDCs (15 PDCs and 8 PDO culture-derived PDC cultures) corresponding to 12 different patients. We compared multiomics data from all subregional derivatives, and analyzed heterogenetic factors that might affect drug responses. B) Organoid architecture resembles primary tumor epithelium. Most organoids had one or more lumens, resembling the tubular structures of the primary tumor. Tumors devoid of a lumen give rise to compact organoids without the lumen. Hematoxylin-eosin (H&E) staining of formalin-fixed paraffin-embedded (FFPE) organoid sections revealed that tumor-derived organoids presented patient-specific heterogeneous morphologies. Organoid (n = 43) bright-field and H&E scale bar = 250 × 10⁻⁶ m. Tissue (n = 12) scale bar = 105 × 10⁻⁶ m. Cell line (n = 21) bright-field scale bar = 70 × 10⁻⁶ m. C) Cytokeratin 20 (CK20) and caudal type homeobox 2 (CDX2), as well as nuclear β-catenin (CTNNB1) and KI-67 (MKI67), were compared between PDOs and parentalt tumors using immunohistochemistry. Also, the expression patterns of MMR genes such as MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 6 (MSH6), and PMS1 homolog 2 (PMS2) were compared between PDOs and matched tumors. PDOs retained similar levels of these markers as the parental tumors. Scale bar = 200 × 10⁻⁶ m.

Table 1. Clinicopathological information of 12 CRC samples.

| SNU number | Sex/age | MSI | p T | p N | p M | PreTx CEA | Tumor size [cm] | Stage | Tumor Loc. | Met Loc. | Rec Loc. | Regimen |
|------------|---------|-----|-----|-----|-----|----------|----------------|-------|------------|----------|----------|---------|
| SNU-4139   | F/38    | MSI-L | 4   | 1   | 1   | 16.7     | 8              | 4     | Sigmoid colon | Liver, lung, bone, p-seeding | N/a      |         |
| SNU-4146   | M/81    | MSI-L | 3   | 1   | 0   | 0.9      | 5.5            | 3     | Sigmoid colon | Xeloda³  |         |         |
| SNU-4351   | F/60    | MSS  | 3   | 0   | 0   | 8.4      | 3.5            | 2     | Sigmoid colon | S-FU(LV)  | Xelox⁶   |         |
| SNU-4374   | F/66    | MSS  | 3   | 0   | 0   | 8.5      | 3.7            | 2     | Descending colon | S-FU(LV)  |         |         |
| SNU-4376A  | M/39    | MSI-H | 4   | 0   | 1   | 12.2     | 12             | 4     | Transverse colon | Liver     | Xelox⁶   |         |
| SNU-4398   | F/83    | MSI-H | 3   | 0   | 0   | 3.3      | 3.1            | 2     | Ascending colon | N/a       |         |         |
| SNU-4631A  | F/75    | MSS  | 4   | 2   | 0   | 1.5      | 8.5            | 3     | Ascending colon | Xelox⁶   |         |         |
| SNU-4646   | M/86    | MSS  | 3   | 1   | 0   | 3.3      | 7              | 3     | Rectum       | Lung      | N/a      |         |
| SNU-4713   | F/86    | MSS  | 3   | 0   | 0   | 3.7      | 5              | 2     | Sigmoid colon | N/a       |         |         |
| SNU-4796   | M/70    | MSS  | 3   | 0   | 0   | 1.3      | 4.5            | 2     | Ascending colon | Xeloda³  |         |         |
| SNU-4813   | M/77    | MSS  | 3   | 0   | 0   | 2.3      | 7.5            | 2     | Sigmoid colon | N/a       |         |         |
| SNU-4849   | M/82    | MSS  | 3   | 0   | 0   | 2.7      | 4              | 2     | Ascending colon | N/a       |         |         |

² MSI indicates microsatellite instability ³ PreTx CEA indicates pretreatment carcinoembryonic antigen ⁴ Met Loc. indicates metastasis location ⁵ Rec Loc. indicates recurrence location ⁶ Xeloda indicates capecitabine ⁷ S-FU(LV) indicates 5-fluorouracil/leucovorin ⁸ Xelox indicates oxaliplatin/capecitabine.

well as PDC lines that retain the original characteristics of the isolated tumors with signs of biological variances among cancer subregional clones from the same origin.

2.2. Subregional Organoids and Cell Lines Recapitulate the Genomic Features with Moderate Intratumor Heterogeneity of Human Colorectal Cancer

Several studies have shown that PDOs and PDCs retain the genomic landscape of the original tumor, including somatic mutations and copy number variations (CNVs).¹⁵c,¹⁷ We performed whole-exome sequencing (WES) on multifocal samples derived from 12 different patients to determine the genomic concordance between parental tumors and its derivatives. For each patient, we sequenced three to four multiregional PDOs, PDCs, a matched tumor tissue and normal mucosa as a control. Although normal mucosa has been shown to contain some cancer-relevant somatic aberrations and transcriptomic features,¹⁸ our study mainly focused on the comparison between subregional clones using variant allele frequencies (VAFs) of driver mutations, and therefore the minor contamination in those normal controls was
considered negligible. Cancer cell evolution may occur as a result of positive clonal selection that is partially sensitive to culture conditions. In order to assure that the observed ITH among different subregional clones was not caused by culture-associated genetic mutations, we performed additional WES of a few subregional clones in their low passages (p1-2, ending with _LP). We compared driver mutations, mutational signatures, CNVs and drug responses of those early passage subregional sets, including two microsatellite stable (MSS) tumors (two patients) and one MSI tumor (one patient). Our results indicate that the observed genetic heterogeneity among subregional clones was well conserved between early and late passages (Figure 2A; Figures S3 and S4, Supporting Information), suggesting that most of the observed ITH is of tumor origin.

WES identified multiple mutations in the samples, including point mutations in putative oncopgenes. The mutational profiles of the samples are summarized in Table S2A,B in the Supporting Information. Two patients (SNU-4376A and SNU-4398) showed features of hypermutation (>10 mutations Mb−1) (Figure 2A). Frequent mutations commonly observed in CRC were well represented in our PDO and PDC sets. These include activating mutations in tumor suppressors such as TP53, APC, and FBXW7, as well as activating alterations in KRAS (codon 12) and PIK3CA (codon 107, 542, 545, 939, 1044, and 1047) (Figure 2A; Table S2A,B, Supporting Information). Genetic alterations in MMR-associated pathways are concomitant with the hypermutated phenotype. The derivatives of two microsatellite instability-high (MSI-H) patients (SNU-4376A and SNU-4398) harbored missense mutations in the POLE gene, with SNU-4398 having an additional point mutation in MSH3, in concordance with their classification as hypermutated CRC. The majority of CRC cases harbored activating mutations in CTNNB1 or inactivating mutations in APC, AXIN2, FBXW7, or FAM123B. Among our non-MSI-H types, we found APC alterations in all but two patients (SNU-4631A and SNU-4796). None carried activating mutations in CTNNB1. The SNU-4631A had a missense mutation in FBXW7. Epigenetic regulation takes a significant role in the initiation and progression of CRC. In our cohort, various genes related to the epigenome were mutated at a high rate, such as ARID1A, KMT2C, KMT2D, and KDM6A, which are commonly altered in CRC. Overall, the mutational spectrums identified in our PDOs and PDCs reflected the genomic features of their parental tumors (Figure 2A; Table S2A,B, Supporting Information).

The mutational concordance of the subregional sets within the coding regions corresponded closely with the matched tumor specimens for both the hypermutated and nonhypermutated patients as expected (median = 0.89 frequency of concordance ranging 0.77 to 0.94 (Figure S3A and Table S3A, Supporting Information)). We analyzed discordant alterations between derivatives and matched tissues to assess the level of ITH in our subregional sets compared to their parent tumor. On average, 5.3% (43/798) of discordant mutations were exclusively present in PDOs and PDCs (Table S3B, Supporting Information). These are expected to be fractional mutations enriched in the subregional lines, which were present at low frequencies in the original tumor. Importantly, the concordance of mutational features in the low passage organoids excludes the possibility of de novo gain of these mutations during culture establishment and subsequent passaging (Figure S3A, Supporting Information). On the other hand, 3.4% (29/844) of discordant mutations were detected solely in tumor tissue (Table S3C, Supporting Information). These tumor-only discordant mutations had a relatively low allelic frequency in the tumor tissue, suggesting the possibility that our multiregion sampling method still misses some subregional mutations due to the limited sample number (n = 3–4). We then looked at the mutational signatures of the subregional sets in comparison to the parent tumors. The most predominant point mutation type in total samples was the C-to-T transition (Table S3D, Supporting Information), closely matching the other large cohort CRC sequencing results. The mutational signature patterns of PDOs and PDCs originating from the same patient were concordant with moderate subregional variations (Figure S3B and Table S3D, Supporting Information).

We also compared the exome-wide CNVs of PDOs and PDCs to the parent tumor tissues, except for the SNU-4351 and SNU-4796 sets, in which the primary tumor specimens and matched normal samples were of insufficient purity to determine the CNVs. Subregional PDOs and PDCs displayed mostly analogous CNV patterns to the parental tumors (Figure S4A–J, Supporting Information). Several CNVs were only observed on selected chromosomes of some subregional clones, such as gain at chromosome 7 and loss at chromosome 14 of the SNU-4146S1-TO sample. We again confirmed that those chromosomal alternations were already present at the initial passages (Figure S4B,C,E, Supporting Information). Our samples displayed comparable CNVs with a much larger clinical cohort as confirmed by TCGA revealed the presence of MYC- and BRCA2-amplified and SMAD4-deleted PDOs and PDCs, as well as a documented gain of the 13q region in the nonhypermutated group (Figure S4L, Supporting Information). Overall, this data confirms that the subregional PDOs and PDCs recapitulate the genomic characteristics of the primary tumor and most of the genomic diversity of CRC. It also shows that our multiregional sampling is a valid method for capturing the genomic ITH of the original tumor, as suggested in previous reports.

2.3. Tracking Evolutionary Histories of 12 Multisampling CRC Sets Revealed Significant Genomic Heterogeneity

We applied the Treeomics algorithm to the multiregional sequencing data to draw evolutionary trajectories. We included PDOs as well as PDCs to determine if the different culture methods affect the mutational aspects. We rectified any possible sequencing artifacts with the Treeomics algorithm to make the mutational patterns of each sample compatible with the topological variant of the evolutionary tree. Based on multiregional WES profiles, Treeomics classified somatic mutations as all (trunk), more than two samples (shared) and a single sample (individual) (Figure 2B–E; Figure S5A–H, Supporting Information). Our cohort involved organoid-derived cell lines (ending with _T), which were suitable for detecting culture-associated mutations. Common driver genes with potential functional mutations, including nonsynonymous single-nucleotide variants, stop-gain SNVs, splicing SNVs, or insertion/deletions (indels) were plotted for analyzing the evolutionary history of each tumor. For instance, SNU-4849 harbored multiple tumor driver mutations, such as...
Figure 2. Subregional analysis of the genomic features of patient-derived organoids and cell lines reveals evolutionary trajectories of 12 CRCs. See also Figures S3–S5 in the Supporting Information. A) Heatmap of mutational variations among the frequently mutated genes in CRC. WES identified multiple somatic mutations including point mutations in putative tumor driver genes in the 12 CRC series, which together consisted of 42 PDOs, 23 PDCs, and 11 tumors samples. The recurrently mutated genes in CRC were recapitulated in the PDOs and PDCs. Derivatives from patient SNU-4376A and SNU-4398 exhibited features of hypermutation (>10 mutations Mb⁻¹). Derivatives are marked with representative colors in accordance with their patient origin. The derivatives ending with _LP indicate subregional clones at initial passage. B,C) Phylogenetic trees demonstrating the evolutionary history of two MSS CRC sets. D,E) Phylogenetic trees illustrating the evolutionary trajectory of two MSI-H CRC sets. Based on the multiregional WES profiles, Treeomics classified somatic mutations as trunk (orange), shared (blue), and individual (green). Numbers on top of each branch indicate the number of acquired variants. Genes that are listed in the CGC are shown in orange. Numbers on the bottom of each branch indicate the estimated support values. The actual photograph of each tumor shows the marked sites from which each subregional derivative was obtained (S1–S4). Scale bars = 1 cm. Multiregional variant allele frequencies (VAFs) are shown as a heatmap under the phylogenetic tree. The three different classes of mutations (trunk, shared, and individual) are indicated by the colored bars on the top. Driver mutations are indicated below each heatmap. The Treeomics statistical settings were identical for all samples (sequencing error rate = 0.005, prior absent probability = 0.5, max absent VAF = 0.05, LOH frequency = 0, false discovery rate = 0.05, false-positive rate = 0.005, and absent classification minimum coverage = 100).
APC (c.4132C > T/p.Q1378*) and TP53 (c.713G > A/p.C238Y), and ARID1A (c.4187_4188del/p.G1396Afs*48), with an allele frequency of \(0.95\) in the trunk, while each of the subregional clones shared the phylogenetic tree with individual mutations of \(0.2\) VAFs (Figure 2B; Table S2B, Supporting Information). This implies that the first hit mainly contributed to the tumorigenesis of epithelial cells, and thereafter the tumor had not progressed. The SNU-4374 tree had a similar shape to SNU-4849, and displayed several driver mutations as well. The SNU-4374 series had several driver mutations as well, such as APC (c.4132C > T/p.Q1378*), TP53 (c.406C > G/p.Q136E) with VAFs of \(0.95\) in the trunk while each of the subregional clones outlined the shape of the tree with individual mutations with VAFs of \(0.3\). Nevertheless, the SNU-4374S2-TO clone had a protruding acquisition of a mutational burden in the KMT2C and SOX9 genes with VAFs of \(0.35\), which suggests one major branch was formed by epigenetic factors (Figure 2C; Table S2B, Supporting Information).

The original tumor tissue of SNU-4146 had a TP53 mutation (c.817C > T/p.R273C) with VAF of 1, which appeared in a relatively early stage of the tumor evolution, and two APC mutations (c.637C > T/p.R213* and c.1312+2T > G/p.X438_splice) with VAFs of 0.51 and 0.43, respectively (Figure S5B, Supporting Information). We observed that VAFs of nonsense APC mutations (c.637C > T/p.R213*) were increased to 0.71 and 0.82 in subregional clones SNU-4146S2-TO and SNU-4146S3-TO, respectively, whereas VAFs of the other two subregional clones remained unchanged. In contrast, the splice site mutation (c.1312+2T > G/p.X438_splice) had decreased VAFs of 0.37 and 0.26 in subregional clones SNU-4146S2-TO and SNU-4146S3-TO, respectively (Figure S5B and Table S2B, Supporting Information). VAFs of the other two subregional clones remained unchanged as well. This analysis revealed that, even though two APC mutations were present in all subregional clones, two major subregional clones were subject to a loss of heterozygosity (LOH), leading to biallelic inactivation of APC. We also made a direct comparison of molecular features between cell lines when established directly from the patient (SNU-4146S4) and adapted from the organoid (SNU-4146S4T). We expected the organoid-derived cell line likely have higher VAFs in driver mutations since they underwent more selective forces than the tissue-derived cell line, yet the VAFs of driver mutations were nearly identical in those cell lines (Table S2B, Supporting Information). The overall mutational concordance of SNU-4146S4 and SNU-4146S4T were also comparable, which was 0.79 and 0.78, respectively (Table S3A, Supporting Information). This suggested that the mutational predisposition of these two cell lines was likely determined by the subregional heterogeneity rather than the different culture methods. We further validated the specific subregional distance between two cell lines by constructing the phylogenetic tree of SNU-4146 patient including both cell lines (Figure S3C, Supporting Information). This analysis revealed that SNU-4146S4 and SNU-4146S4T were adjacent to each other on the phylogenetic tree, which implies they share the majority of subregional mutations. Especially, both cell lines harbored CBWD3 mutation (c.394G > T/p.D132Y) which was absent in SNU-4146S4-TO.

Nearly 50% of the mutations of two MSI-H tumor derivative series (SNU-4376A and SNU-4398) occurred in the same genes. VAFs of major hit mutations including APC, TP53, PIK3CA, and KRAS were relatively low in the MSI-H series (Figure 2D,E). There were discordant driving alterations in APC and TP53, suggesting that hypermutated phenotypes may have been present prior to the LOH, leading to the biallelic inactivation of APC and TP53 and the acquisition of growth-promoting mutations. Another 10 MSS tumor-derived sets share at least one major hit with VAFs of \(0.95\), which indicates that the somatically mutated progenitor cells had been mutually ancestral but then diverged to acquire independent secondary alterations (Figure 2B,C; Figure S5 and Table S2B, Supporting Information).

Multifocal capturing of CRC makes it possible to detect a series of genes that account for not only the initiation but also the evolution of colon tumors. Two MSI patients were not included due to the high tumor mutational burden. To exclude random somatic mutations, only genes found to be mutated in more than three patients were counted. A total of 78 trunk genes that are potentially associated with tumor initiation (Figure S6A and Table S4B, Supporting Information) and 444 shared/individual genes that shaped tumor evolution were mutated (Figure S6B and Table S4B, Supporting Information). Genes that are cross-listed in the Cancer Gene Census (CGC)[28] are highlighted in red. Genes present in the Vogelgram,[27] such as APC, TP53, KRAS, and FBXW7 were exclusively found among the 78 tumor-initiating genes, indicating that in our samples Vogelgram mutations mainly played a role in tumor initiation and had less influence on subregional evolution. Only six genes were present in both tumor initiation and tumor evolution stage: MUC4, MUC16, USP6, BCLAF1, KMT2C, and NOTCH2. Those genes might account for the mutational trajectory of tumor subregional clones.

2.4. Gene Expression Analysis Reveals Substantial Transcriptomic Heterogeneity in 12 Multisampling CRC Tumors

We verified that the patterns of mRNA expression reflect the ITH as well. We validated that there was no mixing of lines before sequencing (Table S1B, Supporting Information), which excluded the potential misinterpretation of mixed sequencing results as transcriptomic ITH. Hierarchical clustering analysis revealed that most of the expression patterns depend on the inter-patient heterogeneity as well as different culture methods. With only a few exceptions, each subregional clone was grouped in accordance with patient origin (Figure 3A). Nevertheless, some subregional clones displayed specifically dispersed patterns. For instance, SNU-4376AS2-TO and SNU-4796S3-TO subregional clones were clustered separately from their parental origin. In addition, none of the subregional clones of the SNU-4374-TO series were clustered at all. PDO subregional clones were evidently separated from PDC subregional clones regardless of spatially diverse clonal capturing (Figure 3A). This indicated that culture conditions had more influence than genetic ITH, and subregional clones cultured in different conditions should not be compared directly by transcriptome analysis.

We also applied our RNA expression data to CMS subtyping by using an R package, CMScaler.[28] A total of 78 RNA expression data from the original tumor tissues, PDOS, and PDCs were subtyped (Figure 3B; Table S5A, Supporting Information). We distributed the samples across the subtypes, with the CMS type 2 (\(n = 38\)) being the most commonly represented. A ma-
Figure 3. Transcriptome analysis validated significant heterogeneity in 12 multisampling CRC tumors. A) Hierarchical clustering analysis of subregional derivatives (n = 70). Euclidean distance (height) of mRNA expressions indicated that the effect of different culture methods as well as interpatient heterogeneity on mRNA expression profiles of each subregional clone was substantial with few exceptions. Derivatives are marked with representative colors in accordance with the patient origin. Unsupervised Ward’s minimum variance method was used as the distance metric for clustering. B) CMS subtyping of subregional derivatives (n = 67) and original tissues (n = 11). Heterogeneous distribution of the CMS types was observed in most of multifocal sampling CRC sets. The original CMS type of the tumor was better retained in PDOs than PDCs. Subtype-specific gene sets with accordant class predictions are marked with symbolic colors for each CMS subtype (yellow: CMS1; blue: CMS2; red: CMS3; green: CMS4). The multifocal subregional clone numbers (S1–S4) are indicated on the top. Unavailable subregional samples are marked with NA. C) Box plot of mRNA heterogeneity scores (DEPTH) showing that SNU-4374, SNU-4376A, and SNU-4796 sets had high transcriptional ITH. Subregional PDOs (n = 43) and PDCs (n = 28) derived from the same patient are marked with representative colors.
jority of 12 CRC sets displayed heterogeneous distribution of the CMS types in their derivatives. For instance, all subregional organoids of SNU-4813 were differently subtyped. Likewise, the SNU-4376AS2-TO subregional clone, which was grouped separately in hierarchical clustering, had a distinct subtype in CMS analysis as well. In addition, CMS subtyping indicated that PDOs better recapitulated the original CMS type of the tumor although PDCs retained moderate subregional heterogeneity. Also, two MSI-H tumors (SNU-4376A-T0 and SNU-4398-T0 series) exhibited different CMS types. While SNU-4398 series were uniformly classified as CMS1 in parallel with its hypermutated and microsatellite unstable nature, SNU-4376A series were subtyped as CMS2 and CMS4.

Next, transcriptomic heterogeneity scores were calculated with the DEPTH algorithm from the R package, and considered as the characteristics of each subregional clones, as described by the author (Table S5B, Supporting Information). We drew the box plot to visualize the degree of dispersion of heterogeneity scores within each patient. This indicated the majority of outliers were of 2D cell lines. For instance, SNU-4351S2 and SNU-4351S4 had significantly higher DEPTH score than the rest of the subregional clones, which should be considered as transcriptomic variances between 2D and 3D cultures rather than subregional heterogeneity. Besides, the number of derivatives in SNU-4376A set (n = 8) was higher than other sets due to the presence of 2D cultures, which might put biased weight on the level of heterogeneity. Based on those evidences, we only counted PDOs to determine transcriptomic heterogeneity within a patient. In parallel with previous hierarchical clustering and CMS subtyping analysis, subregional PDOs from SNU-4374 and SNU-4376A series were spotted apart from each other (Figure 3C). Also, two MSI-H tumors (SNU-4376A and SNU-4398 series) displayed distinct patterns on DEPTH analysis as well. While the SNU-4376A series was close to other MSS tumor derivatives, the SNU-4398 series clustered notably apart from other sets. Also, the degree of intratumor heterogeneity was evidently higher in SNU-4376A than SNU-4398.

Overall, these data validated that the subregional PDOs better recapitulate the transcriptomic characteristics of the primary tumor and most of the transcriptomic diversity of CRC than PDCs. It also demonstrates that our multiregional sampling is an effective method for capturing the transcriptomic ITH of the original tumor.

2.5. Subregional PDOs Reveal Heterogeneous Drug Responses Caused by ITH

We assembled a 24-compound library for drug screening. In total, 38 of 43 tumor organoids and 19 of 23 cell lines from the 12 patients were successfully screened in experimental duplicate, generating >1300 measurements of PDO and PDC-drug interactions. We used complete PDO culture medium when culturing and seeding PDCs for drug treatment in order to minimize the potential variables from different culture conditions.

As a first validation, the grouping of compounds based on their AUC values confirmed a range of responses across the subregional PDOs and PDCs, and identified five major subgroups in accordance with compounds (Figure 4A). One group (I, 1 coordinate) presented relatively high sensitivity to the EGFR/RAS/RAF/MEK/ERK pathway-targeting drugs, while the other group (II, 1 coordinate) exhibited heterogeneous sensitivity. Other groups displayed moderate responses to antimetabolites and topoisoenzyme inhibitors (I, 2 coordinate), or insensitivity (II, 2 coordinate). The final compound set involved phytochemicals, which showed poor responses across all samples (I, 3 and II, 3 coordinates). We mainly focused on the drugs that showed heterogeneous responses across the subregional samples, which includes afatinib, apitolisib, AZD2014, buparlisib, capecitabine, flurouracil, ICG001, irinotecan, MK5108, oxaliplatin, regorafenib, SAHA and trametinib. Importantly, we have included the low-passage subregional clones (ending with LP) to assess whether consecutive passageing alters responses to certain drugs. These subregional clones displayed tight correlation to the corresponding high-passage subregional clones (Figure 4A).

The multifecond samples were likely to be clustered together according to their tumor origin, with variations in several compounds. Within the same cluster, PDCs were grouped adjacent to each other, suggesting that the culture method influences responses to certain drugs. The AUC values calculated from cell lines and organoids were compared using unpaired two-samples t-test to determine if the variation from the different culture methods had affected the responses of certain drugs. The result indicated multiple drugs including Cetuximab, AZD2014, and Olaparib were affected by the different culture methods (Table S6B, Supporting Information), which indicated the integration of such drug responses of 2D cell lines with other heterogeneity factors such as genetic phylogeny or transcriptomic heterogeneity scores (DEPTH) should be avoided.

Previous studies have demonstrated the sidedness of CRC is correlated with the response of certain chemotherapies. We separated the left- and right-sided tumors among our samples, and compared if there are statistically significant variances according to the sidedness. The result revealed the apitolisib and oxaliplatin exhibited better responses in the left-sided CRCs in our samples in agreement with the earlier research (Table S6C and Figure S7A,B, Supporting Information). No correlation between the sidedness and anti-EGFR therapy was found in our samples.

Then, we integrated the effect of the mutational variation of subregional clones on their heterogeneous responses to various drugs. CGC genes that accounted for the initiation (Figure S6A and Table S4A, Supporting Information) and the evolution (Figure S6B and Table S4B, Supporting Information) in our 12 CRC sets were applied to estimate potential gene-drug interactions using multivariate analysis of variance (MANOVA). Among eight genes including TP53 and KRAS being statistically significant (Figure 4A), PABPC1, CEP89, and RAD51B exclusively presented in the tumor evolution stage, which implied a possible association with heterogeneous drug responses (Table S6A, Supporting Information). Several studies have demonstrated left- and right-sided colorectal cancers have different genetics and evolutionary trajectories. We analyzed the sidedness of MSS tumors separately when evaluating tumor initiating and progressing genes with the following standards. When both number and frequency of the mutation are simultaneously higher in right-sided tumors than left-sided tumors, the mutation is assigned to right-sided mutation. If either the number or the frequency is higher, the mutation is designated to no-sided mutation. The rest is assigned
Figure 4. Patient-derived subregional organoids reveal heterogeneous drug responses caused by molecular ITH. A) See also Table S6A–C in the Supporting Information. Heatmap of AUCs of all 24 compounds against 57 CRC derivatives and 8 low-passage organoid samples (ending with _LP). The names of drugs are provided on the right. The derivatives and drugs were k-means clustered, based on their AUC values across the drug panel. The Arabic numerals on the left and Roman numerals on the top of the heatmap are assigned to indicate different subgroups according to k-means clustering. The status of KRAS and TP53, culture types, sidedness, and patient-origins are displayed above the heatmap. B) See also Figure S6C–K in the Supporting Information. Subregional PDOs reveal heterogeneous drug responses caused by mutational ITH. Mutations observed in the trunk were associated with uniform responses among the subregional clones (marked with red lines, poor response; blue lines, good response, 14 trunk genes observed in 8 sets out of 12). Subregional genetic mutations were associated to heterogeneous drug responses (marked with plane figures, 30 cases observed in 11 sets out of 12). Genes that are listed in the CGC are shown in orange, and genes that are associated with sensitivities to certain drugs are shown in blue on the mutational phylogenetic trees. The Treeomics statistical settings were as follows (sequencing error rate $= 0.005$, prior absent probability $= 0.5$, max absent VAF $= 0.05$, LOH frequency $= 0$, false discovery rate $= 0.05$, false-positive rate $= 0.005$, and absent classification minimum coverage $= 100$). Unsupervised Ward’s minimum variance method was used as the distance metric for clustering of mRNA expression and drug responses. C) See also Table S6D in the Supporting Information. The transcriptomic CMS subtyping of subregional PDOs enabled the prediction of capecitabine responses. Multivariate analysis of variance (MANOVA) indicated that subregional clones assigned to CMS2 and CMS4 mostly exhibited poorer responses to capecitabine compared to other subtypes ($p < 0.01$). Symbolic colors are used for each CMS subtype (yellow: CMS1; blue: CMS2; red: CMS3; green: CMS4). The names of sample sets are provided on the right. The multifocal numbers (S1–S4) are indicated on the top. Unavailable samples are marked with NA. D) Culture type-specific protein expression affected the drug response of AZD2014. AKT-mTOR pathway was specifically activated in only PDOs, which resulted in a better response to AZD2014 compared to PDCs (indicated by bold rectangle). E) Clone-specific protein expression influenced the drug response of AZD2014. AKT-mTOR pathway was specifically activated in only a single subregional clone, SNU-437454-TO, which caused a better response to AZD2014 compared to other subregional clones (indicated by bold rectangle).
to left-sided mutation. The tumor initiating genes ($n = 78$) were subclassified with the left-sided ($n = 18$), right-sided ($n = 48$), and no-sided ($n = 12$) mutations (Figure S6A and Table S4A, Supporting Information). The genes accounting for tumor evolution ($n = 444$) were also subclassified with the left-sided ($n = 156$), right-sided ($n = 214$) and no-sided ($n = 74$) mutations (Figure S6B and Table S4B, Supporting Information). This analysis revealed that the majority of tumor initiating mutations were of right-sided, which suggests that right-sided colorectal tumors had accumulated higher mutational burden within the ancestral clone in our samples than left-sided tumors. In contrast, genes that are associated with tumor evolution had no disposition toward the sidedness of the tumor.

We further associated the specific mutational aberrations with drug responses by referring previously documented drug-gene database such as the Drug Gene Interaction Database (DGIdb). We specifically focused the subregional clonality supported by the mutational phylogenetic tree and mRNA expression during the preclinical phase of PDOs since the uneven number of PDCs per each patient depending on the different establishment rates compared to PDOs put biased weight on the level of heterogeneity and possibly shifted the range of AUC values. Thus, for per patient analysis of the drug response, we only included the results from PDOs while PDCs provide sufficient information when integrating entire samples. Genes that are listed in the CCG are shown in orange, and genes that are associated with sensitivities to certain drugs are shown in blue on the mutational phylogenetic trees (Figure 4B; Figure S6C–K, Supporting Information). First, mutations observed in the trunk were associated with uniform responses among the subregional clones (marked with red lines, poor response; blue lines, good response, 14 trunk genes observed in 8 sets out of 12). We also found subregional genetic mutations that are associated to heterogeneous drug responses (marked with plane figures, 30 cases observed in 11 sets out of 12). For instance, all subregional clones in SNU-4139-TO series harbored an identical KRAS mutation, which in-}

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**2.6. Integration of Subregional Transcriptomic and Genomic Analysis Enables Comprehensive Prediction of Heterogeneous Drug Responses**

Although the subregional mutations, CMS subtypes, and protein expressions demonstrated the heterogeneous drug responses clone by clone, more comprehensive prediction of subregional drug responses was achieved by integrating genetic and transcriptional variances. As a first validation, the clustering based on the genetic phylogeny and mRNA expression showed comparable patterns, which substantiated that the genetic ITH is well reflected in heterogeneous gene expression patterns (marked with dotted lines) (Figure 4B; Figure S6C–K, Supporting Information). We then calculated the Pearson correlation coefficient with $p$ values between the transcriptional heterogeneity score (DEPTH) and AUCs of each drug. Among 13 compounds that exhibited heterogeneous drug responses (Figure 4A), the AUC
Figure 5. Integration of subregional transcriptomic and genomic analysis enables comprehensive prediction of heterogeneous drug responses. The Pearson correlation coefficient ($R$) with $p$-values between the transcriptomic ITH score and AUC are indicated on the top of the correlation graph. A value of $p < 0.05$ was considered statistically significant. Confidence intervals are calculated at a confidence level of 0.95 for the parameter and indicated by a shading along with the correlation graph. The specific gene that accounted for unpredicted insensitivity is marked with black circle on the phylogenetic tree. The Treeomics settings were as follows (sequencing error rate $= 0.005$, prior absent probability $= 0.5$, max absent VAF $= 0.05$, LOH frequency $= 0$, false discovery rate $= 0.05$, false-positive rate $= 0.005$, and absent classification minimum coverage $= 100$). A) Among the SNU-4146-TO series, which consists of four wild type KRAS subregional clones, all but SNU-4146S3-TO had unpredictable insensitivity by correlation analysis of DEPTH. This subregional clone harbored a unique NOTCH3 mutation (N1588H), which causes the poor response to afatinib. B) Among the SNU-4351-TO series, only the SNU-4351S3-TO clone was located outside the confidence interval of the correlation plot. This subregional clone harbored a mutated PIK3CG, which is known to cause insensitivity to buparlisib. Unsupervised Ward's minimum variance method was used as the distance metric for clustering of mRNA expression and drug responses.
values of eight compounds including afatinib, buparlisib, apitolisib, SAHA, AZD2014, and flurouracil were in direct proportion to the DEPTH score (Table S6E, Supporting Information). All compounds that were in direct proportion to the DEPTH score were of targeted drugs except for flurouracil. This indicates that the elevated expression of oncogenic signatures in high-ITH tumors diminishes their sensitivity to relevant inhibitors, and the lower expression deviations from the mean expression values is causative factor for the better response of such targeted drugs.

Drug responses to afatinib had a Pearson correlation coefficient of 0.54 to the DEPTH score ($p < 0.0005$). Since the mutant KRAS is already known to cause insensitivity to afatinib, the correlation coefficient was reanalyzed in accordance with the mutational status of KRAS. Whereas the altered KRAS group displayed no correlation between DEPTH score and afatinib response ($R = 0.22, p > 0.05$), the wild type KRAS group presented high correlation ($R = 0.83, p < 0.0005$) (Figure 5A). Among the SNU-4146-TO series, which consists of four KRAS wild type subregional clones, all but SNU-4146S3-TO were positioned near the confidence interval of the correlation graph, which indicates unpredicted insensitivity of SNU-4146S3-TO to afatinib. The DEPTH score alone cannot explain this outlier. However, when genetic mutations were integrated, we found that the SNU-4146S3-TO subregional clone harbored a unique NOTCH3 mutation (N1588H), which has been reported to cause poor response to afatinib (Figure 5A). An analogous integration of mutational phylogeny, mRNA expressions, and drug responses also explained the poorer response to buparlisib in SNU-4351S3-TO clone (Figure 5B). Among the SNU-4351-TO series, only the SNU-4351S3-TO clone was outside the confidence interval of the correlation plot. This subregional clone harbored a mutated PIK3CG gene, which is known to cause insensitivity to buparlisib (Figure 5B).

3. Conclusion

Patient-derived tumor organoids have been widely used and represent a promising new platform for personalized cancer medicine, reflecting their value for basic cancer studies as well as translational research. PDO cultures for in vitro modeling of primary tumors have been applied to CRCs as well as other cancer types. CRCs are associated with heterogeneous clones shaped by a Darwinian selection process. The resulting molecular heterogeneity and phenotypic diversity construct a multifaceted clonal architecture, which forms the basis of drug resistance and metastatic potential. In this study, we have established 12 sets of CRC PDOs and PDCs in order to capture ITH of individual CRC patients. These models confirmed the highly heterogeneous nature of CRC due to the subregional heterogeneity caused by the parallel genetic and transcriptional evolution within a single tumor. Our genetic phylogeny analysis revealed many of the known cancer mutations (e.g., the Vogelgram genes: APC, KRAS, SMAD4, TP53) were found to be shared in the trunk of individual subregional clones, but were rarely specific to sublineages, which suggests that the genetic ITH is probably driven by less characterized cancer mutations. Since we have mixed the DNA from each region of a single tumor, we were not able to construct the phylogenetic trees directly from the tissue, which restricted the valuation of the extent to which the subregional organoid recapitulates the genetic of the subregional tissue. Our work also confirmed that both PDOs and PDCs comparably retain the genomic heterogeneity of primary tumors, yet PDOs are superior preclinical model to PDCs in perspective of capturing the transcriptomic and proteomic heterogeneity. In addition, our multiregional models retaining the molecular ITH from primary tumors enabled the integration of highly prevalent genetic and transcriptional heterogeneity to corresponding heterogeneity of drug responses in CRC.

Taken together, our approach provides a high-fidelity preclinical cancer model to understand the evolutionary trajectories of individual tumors. By capturing the hierarchical clonal structure with live sets of PDOs and PDCs, which recapitulate the histopathological and molecular heterogeneity of human CRC, our data revealed heterogeneous drug responses of CRC in the perspective of the prevalent molecular heterogeneities, and provide potential resolutions to overcome the drug resistance caused by ITH using various molecular layers. It also suggests that single biopsy-based PDOs have intrinsic limitations for predicting patient responses. As a potential solution, our data show the importance of targeting ancestral somatic driver mutations shared by cancer subregional clones in conjunction with comprehensive understandings of transcriptomic and proteomic tumor heterogeneity in order to defy wanted drug resistance caused by ITH.

4. Experimental Section

Nomenclature:

| Institute | Sample Type | Sample Number | Series Number |
|-----------|-------------|---------------|---------------|
| 4139      | Blank       | S1            | 51            |
| 4146      | T: Organoid-Derived 2D Tumor Cell Lines | S2 | 52 |
| 4351      | TO: Patient Tissue-Derived Tumor Organoid | S3 | 53 |
| 4374      | CT: Cancer Tissue | S4 | 54 |
| 4376A     | NT: Normal Tissue | 4398 | 55 |
| 4398      | 4631A       | 4646          | 4713          |
| 4796      | 4913        | 4949          |               |

Data and Material Availability: Raw and processed next-generation sequencing data will be available by request and governed by Ja-Lok Ku. Computational pipelines in this study are available at the public repository (https://github.com/mario2437/CRC_HETERO). Cell lines and organoids generated in this study have been deposited to the KCLB (Korean Cell Line Bank, https://cellbank.snu.ac.kr) biobank and are governed by Ja-Lok Ku.

Ethics Statement: The research protocol was reviewed and approved by the institutional review board of the Seoul National University Hospital (IRB No. 1102-098-357). The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients enrolled in this study.

Sample Collection and Preparation: A total of 45 multifocal specimens of human colorectal tumors were collected from 12 CRC patients who underwent radical resection at Seoul National University Hospital (Seoul, Korea). Tumor tissues were histologically diagnosed by a pathologist as adenocarcinoma. The surgically resected tumor tissue was submerged in Opti-MEM supplemented with 1% penicillin/streptomycin ( GibCO, CA, USA, Cat# 15140-122) and transferred in an ice box directly from the operating room to the laboratory. The resected colon tissue was laterally unfolded in order to expose the neoplasia gland. The multifocal regions of the primary tumor were designated as S1–S4 before preprocessing for the further culture. The minimum lateral distance between two multisite sam-
dance with the labels (S1–S4), tumor tissues were cut into three to four pieces with penicillin/streptomycin (GIBCO, Cat#15140-122) five times. In accordance, primary tumor tissues were photographed and washed in cold PBS Eagle medium/F12, 1% penicillin/streptomycin, Glutamax (10 μg/mL). The tumor tissue was used. Cell lines were established using a previously published protocol.[46] For immunohistochemistry, the middle portion of the tumor tissue was used. Cell lines were established using a previously published protocol.[46] For the control well, the mixture of HISC medium and drug-solvent solution was added. Detailed information about compounds is given in Table 3:

**Tumor Isolation and Culture:** Once the multiregional sites were labeled, primary tumor tissues were photographed and washed in cold PBS with penicillin/streptomycin (GIBCO, Cat# 15140-122) five times. In accordance with the labels (S1–S4), tumor tissues were cut into three to four pieces. Both DNA and RNA were extracted from 0.5 to 0.8 cm³ of tissue materials from each multifocal region. The weight per each tissue material was set to 8–10 mg. Then, the DNA and RNA fragments from each site were mixed with strictly measured quantities (50 μL of 300 ng/μL solution per each site). For immunohistochemistry, the middle portion of the tumor tissue was used. Cell lines were established using a previously published protocol.[46] For the organoid cultures, the remaining tumor tissue from each site was minced into smaller fragments on a sterilized dish on ice and incubated with digestion (5–10 mL) medium supplemented with collagenase II (1.5 mg/mL) (GIBCO, Cat# 17101-015), hyaluronidase (20 μg/mL) (Sigma-Aldrich, MO, USA, Cat# H4306) and Ly27632 (10 × 10⁻³ m) (Selleckchem, TX, USA, Cat# S1049) for 30–60 min depending on the size of tumor piece at 37 °C on a shaker. The mixture was applied to a 100 μm cell strainer (SPL Life Sciences, Gyeonggi-do, Korea, Cat# 93100) to remove large fragments. Tumor cells were centrifuged at 1000 rpm for 3 min, and resuspended in basal culture medium (Dulbecco’s modified Eagle medium/F12, 1% penicillin/streptomycin, Glutamax (10 × 10⁻³ m), 10% FBS). This process was repeated twice to remove debris and digestion solution. Then, red blood cell (RBC) lysis buffer (2–5 mL) (Sigma-Aldrich, Cat# R7757) was added for 1–5 min to remove RBCs. Tumor cells were spun down at 1000 rpm for 3 min, and resuspended in BME (GIBCO, Cat# A14132-02) for seeding in a T-25 flask (Corning, NY, USA, Cat# 533108). Approximately 17–19 BME ( Basement Membrane Extracts) domes (each consisting of BME (50 μL) containing 20 000 cells mL⁻¹) were seeded in a single T-25 flask. The flask was incubated at 37 °C for 10 min. Once BME had solidified, 5 mL of human intestinal stem cell (HISC) medium was added, and cells were incubated in a 37 °C and 5% CO₂ culture incubator. The HISC medium consisted of 40% W/V basal culture medium, 50% W/V L-WRN conditioned medium, 1x B27 (GIBCO, Cat# 17504-044), human EGF (50 ng mL⁻¹) (GIBCO, Cat# PHG0313), human FGF-10 (10 ng mL⁻¹) (Peprotech, Cat# 100–26), nicotinamide (10 × 10⁻³ m) (Sigma-Aldrich, Cat# 72340), N-acetylcycteine (1.25 × 10⁻³ m) (Sigma-Aldrich, Cat# A72635), A83-01 (50 × 10⁻³ m) (Sigma-Aldrich, Cat# SML0788), SB202190 (5 × 10⁻⁹ m) (Sigma-Aldrich, Cat# S7067), prostaglandin E₂ (Sigma-Aldrich, Cat# P5640), and primocin (100 μg mL⁻¹) (GIBCO, Cat# ant-pm-1).

**Colorectal Cancer Organoid Cultures:** Human CRC organoid culture medium was refreshed every three days. CRC organoids were photographed at initial passages (p1–p5). For passaging, the BME dome was mechanically pipetted using TrypLE Express solution (GIBCO, Cat# 12604-021) and organoids were collected in a 15 mL conical tube. The BME dome was mechanically dissociated with intense pipetting, and the tube containing the organoids and BME mixture was incubated at 37 °C for 5–10 min. The organoids were centrifuged at 1000 rpm for 3 min, and the supernatant was aspirated. Once BME was removed, the cell pellet was resuspended with fresh BME, seeded in a T-25 flask and the flask was incubated at 37 °C for 10 min. Once BME was solidified, HISC medium (5 mL) was added to the flask to overlayer the BME dome and cells were incubated in a 37 °C and 5% CO₂ culture incubator.

**Cell Line and Organoid Seeding/Drug Treatment Procedure:** All drug screens were repeated twice. For cell lines, depending on various growth rates, 2–8 × 10⁵ cells mL⁻¹ were seeded in clear-bottomed, white-walled 96-well plates (Corning, Cat# 3903) in complete organoid culture medium (80 μL) in order to minimize the potential variables from different medium conditions that contain fetal calf serum (FCS) and incubated in humidified incubators at 37 °C for 24 h in an atmosphere of 5% CO₂. Organoids were enzymatically and mechanically dissociated into single cells by incubating and pipetting in TrypLE Express solution (GIBCO) for 5 to 10 min. The mixture was spun down at 1500 rpm for 3 min. Once BME was removed, the cell pellet was resuspended with 1:1 mixture of HISC medium and BME (GIBCO). Homogenous BME mixture (40 μL, 20 000 organoids mL⁻¹) was plated in clear-bottomed, white-walled 96-well plates (Corning, Cat# 3903) using E1-ClipTip Electronic Pipettes (Thermo Fisher Scientific, MA, USA). Plates were incubated at 37 °C with 5% CO₂ for 15 min to solidify the BME before adding prewarmed HISC medium (40 mL) to each well using an EpMotion 5070 (Eppendorf Corporate, Hamburg, Germany). 96 h after seeding, drug solution (20 μL) was added to each well. Drugs were serially diluted in DPBS at a ratio of 1:3 from the maximum dose to make 6 dose points. For the control well, the mixture of HISC medium and drug-solvent solution was added. Detailed information about compounds is given in Table 3:

**Adenosine Triphosphate Assay:** After 72 h of drug treatment, CellTiter-Glo (10 μL) (Promega, Cat# G7572) was added to each well for cell lines and CellTiter-Glo 3D Reagent (10 μL) (Promega Cat# G9688) was added to each well for organoids, followed by 5 min of vigorous shaking. After 30 min incubation at room temperature and an additional minute of shaking, luminescence was measured with a Luminoskan Ascent (Thermo Fisher Scientific) over 1000 ms of integration time. Data were normalized to vehicle and area under curve (AUC) values were calculated using R program version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

**Western Blot Analysis:** For cell lines, cells were harvested with a cell scraper after washing with cold PBS three times. For organoids, HISC medium was aspirated and BME dome was washed with cold PBS three times. Then, BME dome was mechanically pipetted using cell recovery solution (5–10 mL) for 2–3 h on ice while shaking and organoids were collected in a tube. Cell pellets were washed with cold PBS three times. Whole protein was extracted with EZRIPA buffer (ATTO Co., Tokyo, Japan) supplied with 1% protease inhibitor (ATTO Co.) and 1% phosphatase inhibitor (ATTO Co.). The volume of lysis buffer was adjusted to the number of cells collected in each vial. The protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Mixtures of equal amounts of protein, SDS buffer (Invitrogen, CA, USA), reducing buffer (Invitrogen), and distilled water was boiled at 98 °C for 10 min. Then, the mixture was loaded on 4–15% Mini-PROTEAN TGX Precast Gels (Bio-RAD,}
Table 3. Detailed information about compounds.

| Drugs          | Company             | Cat No. | Stock quantities [mg] | Solvent | Max concentration [μM] |
|----------------|---------------------|---------|-----------------------|---------|------------------------|
| 5-FU           | Sigma-Aldrich       | S1209   | 200                   | DMSO    | 20 000                 |
| Afatinib (BIBW2992) | Selleckchem       | S1011   | 10                    | DMSO    | 50                     |
| Apotinisib (GDC0860) | Selleckchem     | S2696   | 10                    | DMSO    | 50                     |
| Avastin (Bevacizumab) | Selleckchem      | A2006   | 5                     | DMSO    | 1000 µg mL⁻¹            |
| Bicalulin        | Selleckchem        | S2628   | 50                    | DMSO    | 100                    |
| Belinostat (PKD101) | Selleckchem      | S1085   | 10                    | DMSO    | 100                    |
| Buparlisib (BKM120) | Selleckchem      | S2247   | 10                    | DMSO    | 100                    |
| Camptosar (Irinotecan Hydrochloride) | Selleckchem | S2217   | 25                    | DMSO    | 100                    |
| Cefoxitin        | Selleckchem        | S1848   | 50                    | DMSO    | 100                    |
| Cyclosporine     | Selleckchem        | S1146   | 10                    | EtOH    | 50                     |
| Eloxatin (Oxaliplatin) | Sigma-Aldrich     | S1224   | 50                    | DMSO    | 50                     |
| Erbitux (Cetuximab) | Selleckchem     | A2000   | 5                     | DMSO    | 1000 µg mL⁻¹            |
| Genistein        | Selleckchem        | S1342   | 100                   | DMSO    | 100                    |
| ICG-001         | Selleckchem        | S2662   | 25                    | DMSO    | 100                    |
| Lonsurf (TAS-102) | Selleckchem       | S8539   | 25                    | DMSO    | 100                    |
| MK-5108         | Selleckchem        | S2770   | 10                    | DMSO    | 100                    |
| Olaparib (LYNPARZA) | Selleckchem      | S1060   | 25                    | DMSO    | 50                     |
| Resveratrol      | Selleckchem        | S1396   | 100                   | DMSO    | 200                    |
| Stivagar (Regorafenib) | Selleckchem     | S1178   | 10                    | DMSO    | 100                    |
| Triflurin (GSK1120212) | Selleckchem     | S2673   | 10                    | DMSO    | 50                     |
| Vistusertib (AZD2014) | Selleckchem     | S2783   | 10                    | DMSO    | 50                     |
| Vorinostat (SAHA) | Selleckchem       | S1047   | 200                   | DMSO    | 50                     |
| Wellcovorin (Leucovorin Calcium) | Selleckchem | S1236   | 50                    | ddH2O   | 10 000                 |
| Xeloda (Capecitabine) | Selleckchem       | S1156   | 50                    | DMSO    | 1000                  |

Table 4. Primary antibodies and the dilution factors used in this study.

| Target                        | Company                    | Cat No. | Dilution |
|-------------------------------|----------------------------|---------|----------|
| Rabbit monoclonal anti-mTOR  | Cell Signaling Technology  | 2983; RRID:AB_2105622 | 1:1000   |
| Rabbit monoclonal anti-pmTOR (Ser2448) | Cell Signaling Technology | 2971; RRID:AB_130970 | 1:1000   |
| Mouse monoclonal anti-panAKT | Cell Signaling Technology  | 2970; RRID:AB_1147620 | 1:1000   |
| Rabbit monoclonal anti-pAKT (Thr308) | Cell Signaling Technology | 86758; RRID:AB_2800089 | 1:1000   |
| Rabbit polyclonal anti-PTEN  | Cell Signaling Technology  | 9552; RRID:AB_10694066 | 1:1000   |
| Rabbit polyclonal anti-β-tubulin | Cell Signaling Technology | 2146; RRID:AB_2210545 | 1:2000   |

CA, USA) and blotted at 50 V for 2 h. Proteins were then transferred to Trans-Blot Turbo Transfer Pack (BIO-RAD) using Trans-Blot Turbo Transfer System V1.02 machine (BIO-RAD) at 2.5 Amp and 25 V. The membrane was incubated in 2.5% skim milk (BD biosciences, NJ, USA) containing 1 × 10⁻³ mM MgCl₂, 10% TBS buffer, and 0.5% Tween 20 (VWR Life Science, PA, USA) for an hour at room temperature. Primary antibodies and the dilution factors used in this study are summarized in Table 4.

After washing with 2.5% skim milk three times, mouse or rabbit IgG secondary antibody (Jackson ImmunoResearch Labs, PA, USA) conjugated with peroxidase diluted in 2.5% skim milk solution (1:5000) was applied to membrane. SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) was applied to the membrane which was then exposed to Fuji RX film (Fujifilm, Tokyo, Japan) for 1–10 min.

H&E Staining and Immunohistochemistry: Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Then, tissues were sectioned at 4 μm thickness. For organoids, the BME dome was mechanically scraped with a pipet tip. Cold PBS (10 mL) was added to collect dissociated BME domes and transferred to a 15 mL conical tube. After 15 s centrifugation at 100 rpm, the supernatant was aspirated. This procedure was repeated until the BME gel was visibly removed. Care was taken not to destroy the original structure of the organoids. Collected organoids were embedded in 2% agarose gel (INTRON Biotechnology, Seongnam, Korea). Solidified agarose gel was fixed in 10% formalin for 30 min at room temperature and sectioned at 4 μm thickness. Sections were subjected to H&E as well as immunohistochemical staining. For immunohistochemistry, the Ventana BenchMark XT Staining system (Ventana Medical Systems, AZ, USA) and OptiView universal DAB kit (Ventana, Cat# 760-700) were used according to the manufacturers’ protocol. Briefly, the sectioned tissues or organoids were mounted on a positively charged glass microscope slide. Slides containing the section were baked for 2 h in a 60 °C oven. Then, the slides were deparaffinized with EZ prep solution (Ventana) at 76 °C for 4 min. To increase renaturation of protein molecules and antibody accessibility, pH 8.4 cell conditioning 1 (CC1) buffer (Ventana) was applied to the slide at 100 °C for 24 min followed by 3% H₂O₂ solution treatment.
Table 5. Antibodies used for immunohistochemistry.

| Target                                | Company                | Catalog          | Dilution |
|---------------------------------------|------------------------|------------------|----------|
| Mouse monoclonal anti-Cytokeratin 20  | Santa Cruz Biotechnology | Cat# sc-271883  | 1:200    |
| Mouse monoclonal anti-CDX2            | BioGenex               | Cat# AM392       | 1:300    |
| Mouse monoclonal anti-β-catenin       | BD Biosciences         | Cat# 610153      | 1:800    |
| Rabbit polyclonal anti-Ki-67          | Abcam                  | Cat# ab15580     | 1:500    |
| Mouse monoclonal anti-MLH1            | Santa Cruz Biotechnology | Cat# sc-56161    | 1:500    |
| Mouse monoclonal anti-MSH2            | Santa Cruz Biotechnology | Cat# sc-137015   | 1:500    |
| Mouse monoclonal anti-MSH6            | Santa Cruz Biotechnology | Cat# sc-271080   | 1:500    |
| Mouse monoclonal anti-PM52            | Santa Cruz Biotechnology | Cat# sc-25315    | 1:500    |

...at 37 °C for 4 min. Primary antibodies were applied at 37 °C for 16 min. Antibodies used for immunohistochemistry are summarized in Table 5. HK linker and HRP multimer solution (Ventana) was applied at 37 °C for 8 min. Then, 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen (Ventana) was applied at 37 °C for 8 min followed by hematoxylin and bluing reagent treatment at 37 °C for 8 min. The stained positive tissue control was examined with hematoxylin to ascertain that all reagents were functioning properly.

**Immunocytochemistry:** The BME dome was mechanically scrapped with a pipet tip. Cold PBS (10 ml) was added to collect dissociated BME domes and transferred to a 15 ml conical tube. After 100 rpm, 15 s centrifugation, the supernatant was aspirated. This procedure was repeated until the BME gel was visibly removed. Care was taken not to destroy the original structure of the organoids. Then, organoids were fixed and permeabilized with BD Cytofix/Cytoperm (BD Science, CA, USA). After cells were washed with washing solution (BD Science), DPBS containing 2% FBS (GE Healthcare Life Sciences, Buckinghamshire, UK) was applied for an hour. After organoids were washed with cold DPBS, CD33 antibody (Abcam, Cambridge, UK) (1:400) diluted in 0.05% of PBS.T was applied for 1.5 h at room temperature. Thereafter, cells were washed with 0.05% of PBS.T, and Alexa 488 and Alexa 568 secondary antibodies (Thermo Fisher Scientific, 1:500) diluted in 0.05% of PBS.T were applied for an hour at room temperature. DAPI (1:100) and rhodamine-conjugated phalloidin (Sigma-Aldrich, 1:10) were diluted in distilled water and applied for 30 min at room temperature. Cells were washed with DPBS three times and placed under a confocal microscope. LSM800 Confocal Laser Scanning Microscope and ZEN software (Carl Zeiss, Oberkochen, Germany) were used to analyze images. Digital resolution, scan speed, and the number of pictures averaged were set to 1024 x 1024, 40 s per one channel, and 8 pictures, respectively. Disperse magnifications were used in accordance with growth patterns and sizes of cells. The intensity of each channel was fixed for comparing target protein expression between samples.

**Whole-Exome Sequencing:** Whole-exome capture was performed on all samples with the SureSelect Human All Exon V5 Kit (Agilent Technologies, Tokyo, Japan). The captured targets were subjected to sequencing using HiSeq 2500 (Illumina, San Diego, CA, USA) with 100x coverage depth for the DNA extracted from organoids and cell lines, and 200x coverage depth for the DNA extracted from the tissue samples. The sequence data were processed through an in-house pipeline. Briefly, paired-end sequences were first mapped to the human genome, where the reference sequence was UCSC assembly hg19 (original GRCh37 from NCBI, Feb. 2009) using the mapping program BWA (version 0.7.12), and generated a mapping result file in BAM format using BWA-MEM. Then, Picard-tools (ver.1.130) were applied in order to remove PCR duplicates. The local realignment process was performed to locally realign reads with BAM files reducing those reads identically matched to a position at the start into a single one, using MarkDuplicates.jar, which required reads to be sorted. By using the Genome Analysis Toolkit, base quality score recalibration (BQSR) and local realignment around indels were performed. Haplotype Caller of GATK (GATKv3.4.0) was used for variant genotyping of each sample based on the BAM file previously generated (SNP and short indel candidates were detected). Somatic mutations were identified by providing the reference and sequence alignment data of tumor tissues or organoids to the MuTect2 (involved in GATK v3.8.0) with default parameters using tumor-normal mode. The matched normal tissue was not available for SNU-4376 series, and peripheral blood mononuclear cells (PBMCs) were used for somatic mutation calling. Those variants were annotated by Snippy v4.10 to vcf file format, filtering with dbSNP for the version of 142 and SNPs from the 1000 Genome Project. Then, Snippy was applied to filter additional databases, including ESP6500, ClinVar, dbNSFP 2.9. Mutational signatures were evaluated using the Mutational Patterns R package, release 3.6.1[43] to configure distinct footprints in genomic context for all somatic SNVs and evaluate a multitude of mutational patterns of base substitution in tumor tissues and matched cell lines/organoids.

**Analysis of CNVs:** For the detection of CNVs and LOH from exome sequencing data, the ExomeCNV package was employed in the R program.[48] The final log ratio of depth of coverage was determined by the number of bases targeted by exome sequencing (targeted base) and the number of bases actually sequenced (mapped). CNV calls were expressed as 1, 2, and 3, which indicated deletion, normal, and amplification, respectively.

**Construction of Evolutionary Trees:** The evolutionary trajectories of twelve CRC cases were traced using the Treeomics algorithm[26] and WES data of multiregion samples. The Treeomics settings were identical for all samples (sequencing error rate = 0.005, prior absent probability = 0.5, max absent VAF = 0.05, LOH frequency = 0, false discovery rate (FDR) = 0.05, false-positive rate = 0.005, and absent classification minimum coverage: 100). Input parameters included read depths of both mutant and coverage genes, gene symbols, chromosomal coordinates, and substitution patterns. The Treeomics algorithm also provided likely driver genes mutations and built-in Cancer Gene Census list. Although the MUC6 gene was included in Cancer Gene Census genes, the number of mutations in the MUC6 gene outnumbered other genes. Consequently, the structure of the evolutionary trees was largely affected by the MUC6 variations. To eliminate potential bias, mutations of the MUC6 gene were excluded from the input data. Sequencing artifacts were automatically adjusted by the Treeomics default setting to confirm the topological configuration of the evolutionary tree was compatible with the mutational patterns. Subregional clone analysis was conducted by adding “-u” parameter to input commands.

**Analysis of RNA Sequencing:** Total RNA was isolated from cell lysate using TRIzol (Qiagen) and Qiagen RNeasy kit (Qiagen, Cat# 74104). Paired end sequencing reads of cDNA libraries (101 bp) generated from a NovaSeq6000 instrument were verified with FastQC v 0.11.7. For data preprocessing, low quality bases and adapter sequences in reads were trimmed using Trimmomatic v0.38. The trimmed reads were aligned to the human genome (UCSC hg19) using HISAT v2.1.0, a splice-aware aligner. Then, transcript assembly of known transcripts, novel transcripts, and alternative splicing transcripts was processed by StringTie v1.3.4d.[49] Based on the result, expression abundance of transcript and gene were calculated as read count or FPKM value (fragments per kilobase of exon per million fragments mapped) per sample. Consensus molecular subtype (CMS) of...
each sample was analyzed by R package, CMSCaller.\[29\] Raw readcounts were used as a direct input with “RNA-seq = True” setting. Heterogeneity scores were calculated with the DEPTH algorithm from the R package\[31\] with default setting. Cel-ID package\[30\] was also applied for authentication of lines. FASTQ files were processed from the RNA-seq to call the variants, which provided sequencing depth and allele frequency information. Briefly, the trimmed reads using Trimomatic v0.38 were aligned to the human genome (UCSC hg19) using STAR 2.6.0c. Then, GATK pipeline v4.2.0.0 including STAR 2-pass mapping, Picard MarkDuplicate, Split “N” Trim, Realignment, and Base recalibration steps was applied. Haplotype Caller was used for variant genotyping of each sample based on the BAM file previously generated.

Statistical Analysis: For hierarchical cluster analysis on a set of dissimilarities, each object was assigned to its own cluster, which an algorithm proceeds through iteratively. Two of the most similar clusters were joined at each stage until there was a single cluster. Distances between clusters were recomputed at each stage by the Lance–Williams dissimilarity update formula according to the particular clustering method being used. Clustering methods included: Ward’s minimum variance method, k-means method, complete linkage method, and single linkage method. For the CMS analysis, readcounts were used from the RNA-seq data as indicated from the R package, CMSCaller.\[29\] The data were presented with p-values with FDR. The heterogeneity score was presented with box plot indicating minimum score, first (lower) quartile, median, third (upper) quartile, and maximum score. Outliers from the heterogeneity score were kept due to the transcriptomic variance between 2D and 3D cultures. A multivariate analysis of variance (MANOVA) model was applied to the drug response data matrix with various factors including the mutational status and CMS subtypes. The mutational status of 20 selected genes were tested to AUC values across 24 drugs (n = 57). The data was presented with Pillai’s trace score, approximate F value and p-value for each of the gene–drug pairs. The four CMS subtypes were tested to AUC values across 24 drugs as well (n = 57). The data were presented with sum of squares, mean square, F value, and p-value for each drug. Unpaired two-samples t-test was performed in order to determine compounds in which its AUC was affected by the two different culture types (2D vs 3D) and the sidedness of the tumor (left vs right-sided) (n = 69). The result was presented with p-value, confidence interval, estimate mean, and standard error for each drug. A value of p < 0.1 was considered statistically significant. Outliers from the drug screening results were kept since it can be derived from the insensitivity for specific drug. The linear dependence between DEPTH scores and AUC values for 24 drugs was calculated with Pearson correlation (n = 57). Confidence intervals were calculated at a confidence level of 0.95 for the parameter. A value of p < 0.05 was considered statistically significant unless otherwise specified. All statistical analysis was performed in R program version 4.0.4 unless otherwise specified.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
S.-C.K., S.-Y.J., and J.-L.K. conceptualized the paper. S.-C.K., J.W.P., M.J.K., S.-Y.J., and J.-L.K. prepared the methodology and S.-C.K. worked with the software. S.-C.K. and H.-Y.S. were involved in validation and visualization. S.-C.K. carried out formal analysis and data curation. Investigation was carried out by S.-C.K., H.-Y.S., J.-H.P., G.-H.K., and J.O.L. J.W.P., H.-Y.S., M.J.K., J.-H.P., G.-H.K., J.O.L., Y.-K.S., J.M.B., S.-Y.J., and J.-L.K. were involved in writing, review, and editing. J.W.P., B.-K.K., S.-Y.J., and J.-L.K. did the supervision. S.-Y.J. and J.-L.K. were involved in project administration. S.-C.K., Y.-K.S., and J.-L.K. were involved in funding acquisition.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
colorectal cancer, drug responses, intratumor heterogeneity, patient-derived organoid

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