Multiomic molecular characterization of the response to combination immunotherapy in MSS/pMMR metastatic colorectal cancer

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ABSTRACT

Background Immune checkpoint inhibitor (ICI) combinations represent an emerging treatment strategy in cancer. However, their efficacy in microsatellite stable (MSS) or mismatch repair-deficient (pMMR) colorectal cancer (CRC) is variable. Here, a multiomic characterization was performed to identify predictive biomarkers associated with patient response to ICI combinations in MSS/pMMR CRC for the further development of ICI combinations.

Methods Whole-exome sequencing, RNA sequencing, and multiplex fluorescence immunohistochemistry of tumors from patients with MSS/pMMR CRC, who received regorafenib plus nivolumab (REGONIVO) or TAS-nivolumab (TASNIVO) in clinical trials were conducted. Twenty-two and 23 patients without prior ICI from the REGONIVO and TASNIVO trials were included in this study. A biomarker analysis was performed using samples from each of these studies.

Results The epithelial-mesenchymal transition pathway and genes related to cancer-associated fibroblasts were upregulated in the REGONIVO responder group, and the G2M checkpoint pathway was upregulated in the TASNIVO responder group. The MYC pathway was upregulated in the REGONIVO non-responder group. Consensus molecular subtype 4 was significantly associated with response (p=0.035) and longer progression-free survival (p=0.006) in the REGONIVO trial. CD8+ T cells, regulatory T cells, and M2 macrophages density was significantly higher in the REGONIVO trial responders than in non-responders. Mutations in the POLE gene and patient response were significantly associated in the TASNIVO trial; however, the frequencies of other mutations or tumor mutational burden were not significantly different between responders and non-responders in either trial.

Conclusions We identified molecular features associated with the response to REGONIVO and TASNIVO, particularly those related to tumor microenvironmental factors. These findings are likely to contribute to the development of biomarkers to predict treatment efficacy for MSS/pMMR CRC and future immunotherapy combinations for treatment.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune checkpoint inhibitor combinations are emerging treatment strategies in cancer. However, biomarkers of response in microsatellite stable mismatch repair-deficient (MSS/pMMR) colorectal cancer have not been identified.

WHAT THIS STUDY ADDS

⇒ We identified molecular features associated with the response to regorafenib plus nivolumab or TAS-116 plus nivolumab combinations. Specifically, activation of genes in the epithelial-mesenchymal transition pathway and consensus molecular subtype 4 enrichment were predictive biomarkers in the REGONIVO trial.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our analyses could lead to the further development of biomarkers for MSS/pMMR colorectal cancer and additional combinations of immunotherapies for treatment.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths worldwide.1 Increasing numbers of immune checkpoint inhibitors (ICIs) have become available as a treatment option for various malignant tumors.2–7 However, for metastatic CRC, the efficacy of ICIs is limited to patients with microsatellite instability-high or mismatch repair-deficient tumors, and the majority of microsatellite stable (MSS) or mismatch
repair-proficient (pMMR) CRC tumors do not respond to treatment with individual ICIs.8–11 The limited effect of ICIs on MSS and pMMR CRC may be attributed to a low neoantigen load and few tumor-infiltrating lymphocytes, which prevents a robust immune response. Numerous immunosuppressive cells, such as regulatory T (Treg) cells and tumor-associated macrophages, may also infiltrate the tumor microenvironment to prevent antitumor activity.12–14 To overcome these resistance mechanisms, several immunotherapy combinations have been evaluated for MSS or pMMR CRC15 16; however, most have been largely ineffective. Combinations of the MEK inhibitor cobimetinib and the programmed death-ligand 1 (PD-L1) inhibitor atezolizumab as well as the multikinase inhibitor lenvatinib and the programmed cell death protein 1 (PD-1) inhibitor pembrolizumab as salvage therapy have failed to exhibit a survival benefit compared with the standard of care in phase III trials.15 16 Further development of ICI combinations for MSS or pMMR CRC is necessary.

Previously, we conducted two investigator-initiated trials of ICI combinations of the PD-1 inhibitor nivolumab with drugs expected to activate the immune response, namely, the multikinase inhibitor regorafenib plus nivolumab (REGONIVO) and the HSP90 inhibitor TAS-116 (pimtespib) plus nivolumab (TASNIVO) for MSS or pMMR CRC, which demonstrated efficacy in a limited number of these patients.17 18 These findings highlighted the need to identify biomarkers to identify patients who would benefit from such combinations and to understand the mechanisms through which this efficacy was achieved for the further development of ICI combinations. To identify predictors of response to ICI combinations in patients with MSS or pMMR CRC, we characterized tumors from patients who received REGONIVO or TASNIVO in clinical trials using whole-exome sequencing (WES), RNA sequencing, and multiplex fluorescence immunohistochemistry (mIHC). By applying this multiomics approach, we characterized these tumors at the molecular level and identified molecular features that may contribute to the development of predictive biomarkers and future immunotherapy combinations.

**METHODS**

**Patients**

The eligibility criteria for this study were as follows: (1) enrollment in a phase Ib trial of REGONIVO (EPOC1603)17 or a phase Ib trial of TASNIVO (EPOC1704)18; (2) patients with MSS or pMMR CRC; and...
and mIHC staining. Most of the samples were primarily FFPE samples were subjected to WES, RNA sequencing, and Cufflinks. The DNA in the resulting libraries was subjected to next-generation sequencing, and 150 bp was sequenced from both ends on a NovaSeq 6000 (Illumina) to produce paired-end reads. Paired-end sequencing reads with masked nucleotides with quality scores less than 20 were aligned to the hg38 reference genome using BWA-MEM (http://bio-bwa.sourceforge.net/) and Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Somatic synonymous and non-synonymous mutations were called using our in-house caller and two publicly available mutation callers: Mutect2, as part of the Genome Analysis Toolkit (https://gatk.broadinstitute.org/hc/en-us), and VarScan2 (http://varscan.sourceforge.net/). Mutations meeting any of the following criteria were discarded: tumor sample variant allele frequency <0.05; mutant read number in the germline control samples of >2; mutations detected in only one strand of the genome; or the variant present in the normal human genome in either the 1,000 Genomes Project data set (https://www.internationalgenome.org/) or our in-house database. Gene mutations were annotated using SnpEff (http://snpeff.sourceforge.net). Tumor mutational burden (TMB) was defined as the total number of mutations per megabase in the WES bait region. Targeted gene panel analysis data (Oncomine Cancer Research Panel, Thermo Fisher) were used for complementarity when WES data were not available.

RNA sequencing
Total RNA was extracted from FFPE tumor samples using the RNaseq FFPE Kit (QIAGEN). Ribosomal RNA deple- tion was performed using the NEBNext rRNA Depletion Kit (New England Biolabs). RNA integrity was assessed using TapeStation (Agilent Technologies). To exclude the degraded RNA, RNA of sufficient integrity was used for RNA sequencing (RNA-seq) with an NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs). Prepared RNA libraries were subjected to next-generation sequencing on a NovaSeq 6000 (Illumina) to produce paired-end sequencing reads. For RNA-seq data expression profiling, paired-end reads were aligned to the hg38 human genome and quantified using TopHat2 (https://github.com/infphilo/tophat) and Cufflinks (https://github.com/cole-trapnell-lab/cufflinks).

Gene Set Enrichment Analysis (GSEA) was performed using GSEA V.4.3.2 (https://github.com/GSEA-MSigDB). Genes were ranked based on a log2-fold change in expression and gene enrichment scores were calculated based on the rank of the genes and gene sets. Gene sets from the Molecular Signatures Database V.7.2.

Consensus molecular subtypes (CMSs) were evaluated as described previously.

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>REGONIVO</th>
<th>TASNIVO</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
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<tr>
<td>Median, years (range)</td>
<td>57.5 (41–77)</td>
<td>61 (32–77)</td>
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<tr>
<td>≥65, n (%)</td>
<td>6 (27.3)</td>
<td>10 (43.5)</td>
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<td>Sex, n (%)</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>12 (52.2)</td>
</tr>
<tr>
<td>Female</td>
<td>6 (27.3)</td>
<td>11 (47.8)</td>
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<td>ECOG PS, n (%)</td>
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<tr>
<td>0</td>
<td>22 (100)</td>
<td>19 (82.6)</td>
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<td>1</td>
<td>0 (0)</td>
<td>4 (17.4)</td>
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<td>Previous treatment regimens, n (%)</td>
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<tr>
<td>≥2</td>
<td>22 (100)</td>
<td>22 (95.7)</td>
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<td>Primary tumor location, n (%)</td>
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<td></td>
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<tr>
<td>Right side</td>
<td>14 (63.6)</td>
<td>15 (65.2)</td>
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<tr>
<td>Left side</td>
<td>3 (13.6)</td>
<td>14 (60.9)</td>
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<td>Metastatic sites, n (%)</td>
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<tr>
<td>Liver</td>
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<td>Lung</td>
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<td>15 (65.2)</td>
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<td>Peritoneum</td>
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<td>11 (47.8)</td>
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<td>Number of metastatic organs, n (%)</td>
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<tr>
<td>1</td>
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<tr>
<td>≥2</td>
<td>18 (81.8)</td>
<td>16 (69.6)</td>
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</table>

(3) no ICI therapy prior to trial enrollment. The detailed methods of these trials have been previously reported. The study was conducted in accordance with the Declaration of Helsinki. The results are publicly available on the official website of National Cancer Center Hospital East, and the research subjects were provided with an opportunity to decline participation.

Samples
Tissues were obtained from patients prior to the administration of the investigational treatment and were formalin-fixed and paraffin-embedded (FFPE). The FFPE samples were subjected to WES, RNA sequencing, and mIHC staining. Most of the samples were primary tumors that were surgically resected before patient enrollment, and all tumor samples were collected prior to any ICI combination therapy, with none collected after any immunotherapy; additional details are provided in online supplemental table S1. Peripheral blood mononuclear cells or normal colon tissue were also used as germ line controls.
Multiplex immunohistochemistry and PD-L1 staining

FFPE tumor tissue blocks were sliced into 4 mm-thick sections and placed on adhesion microscope slides (Matsunami). The tissue slides were deparaffinized and rehydrated prior to mIHC staining. Antigen retrieval and staining were performed using Opal 7-Color IHC Kits (AKOYA Biosciences) according to the manufacturer’s protocol. CD3 (Clone SP7; Abcam), CD8 (Clone C8/144B; Dako), CD206 (Clone CL0387; Invitrogen), CD11b (Clone D6×1N; Cell Signaling Technology), FOXP3 (Clone 236A/E7; Abcam), PDGFRα (Clone D1E1E; Cell Signaling Technology) and Cytokeratin (Clone AE1/AE3; Abcam) staining was examined and images were acquired using the Vectra 3 System (PerkinElmer). Images were exported using inForm Tissue Analysis Software (AKOYA Biosciences). Cell density and expression percentage of specific protein were calculated based on the average of at least three regions of interest (682 μm×510μm/region) using HALO image analysis software (Indica Labs).

For PD-L1 staining using the anti-PD-L1 28–8 antibody, the combined positive score (CPS) was assessed by a pathologist (TKu) and defined as the percentage of total tumor cells (including tumor cells, lymphocytes, and macrophages) multiplied by 100 in the REGONIVO trial. In the TASNIVO trial, CPS was measured by the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies).

Outcomes and statistics

Patients experiencing a clinical benefit (responders) were defined as those who achieved a complete response (CR), partial response (PR), or stable disease (SD) lasting more than 6 months as evaluated by Response Evaluation Criteria in Solid Tumors V.1.1 criteria. Progression-free survival (PFS) was defined as the time from registration for clinical trials to disease progression or death (for any reason). Overall survival (OS) was defined as the time from registration to death (for any reason). Quantitative data are presented as the median and range. The Mann-Whitney U and χ² tests were used for comparisons between continuous and categorical variables, respectively. PFS and OS were estimated using the Kaplan-Meier method, and HRs and CIs were estimated using a Cox
proportional hazards model. All statistical analyses were performed using SAS Release V.9.4 (SAS Institute).

RESULTS

Patients

Twenty-four and 25 patients from the REGONIVO and TASNIVO trials, respectively, with MSS or pMMR CRC without prior ICI treatment, met the eligibility criteria for this study (figure 1). We successfully performed RNA-seq and WES and obtained gene panel and mIHC data for 22 and 23 patients in the REGONIVO and TASNIVO trials, respectively (figure 1). Patients with left-sided tumors were observed more frequently in the REGONIVO trial (86.4%), whereas those with right-sided tumors were observed more frequently in the TASNIVO trial (60.9%) (table 1). Ten patients in each of the REGONIVO (45.5%) and TASNIVO (43.5%) had liver metastasis (table 1). Thirteen of 22 (59%) patients in the REGONIVO trial and 7 of 23 (30%) patients in the TASNIVO trial were classified as responders (CR, PR, and SD ≥ 6 months) (figure 2). In this study, all samples used for WES, RNA-seq, and mIHC staining were obtained from patients prior to ICI therapy and were FFPE.

Molecular features associated with clinical outcomes

Mutational features

We analyzed the differences in the mutational profiles between responders and non-responders in each trial using WES (figure 3 and online supplemental table S2). The mutational landscape of each trial cohort was comparable with that of previous reports. We observed a significant association between POLE (DNA polymerase epsilon) mutations and positive response in the TASNIVO trial (p=0.015), in which two cases with POLE missense mutations achieved PR. One out of two patients harboring POLE mutations exhibited an extremely high TMB (78 mutations/Mb). The TMB of the other patient could not be analyzed because no samples were available. The frequencies of the other representative gene mutations in CRC, including KRAS, ERBB2, BRAF, PIK3CA, TP53, ATM, APC, AXIN2, LRP5, TCF7L2, SMAD2/3/4, ARID1A, and FBXW7, were not significantly different between responders and non-responders in either trial.
(figure 3 and online supplemental table S2). TMB was not associated with the response to either of the ICI combinations even after excluding POLE cases (online supplemental figure S1).

### Transcriptomic features

To find the difference in gene expression and upregulated signal between responders and non-responders, we next performed transcriptome analysis and GSEA on the cohorts in both trials. These analyses revealed pathways associated with the response to each combination therapy. Specifically, upregulation of the epithelial-mesenchymal transition (EMT) pathway was observed in the REGONIVO responder group (figure 4). The expression of representative EMT pathway genes, such as TGFB3, VIM and FN1, were upregulated in the responder group (online supplemental figure S2B). Notably, genes related to cancer-associated fibroblasts (CAFs) were also upregulated (online supplemental figure S2B). In addition, genes related to the inflammatory response were upregulated in the REGONIVO responder group, and we observed a significant upregulation of immune-related genes such as STAT3 (online supplemental figure S2A). Importantly, we also observed upregulation of the PDGFRA gene, a known target of regorafenib, in the REGONIVO responder group (online supplemental figure S2A).

Upregulation of the MYC pathway was observed in the REGONIVO non-responder group (figure 4). Upregulation of genes associated with the G2M checkpoint pathway was observed in the TASNIVO responder group. Additionally, upregulation of PI3K_AKT_MTOR pathway genes was observed in the responder group, and AKT1 and HRAS expression were significant upregulated (online supplemental figure S2C).

### CMS classification of CRC

Given the results of our transcriptomic analysis, we next sought to elucidate the differences in CMS classification using RNA-seq data. CMS classification was possible in 20 of 22 patients in the REGONIVO trial and 21 of 23 patients in the TASNIVO trial. CMS1, CMS2, CMS3, and

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**Figure 4** GSEA gene sets enriched in responders and non-responders in the REGONIVO and TASNIVO trial. ES, enrichment score; FDR, false discovery rate; GSEA, Gene Set Enrichment Analyses; NES, normalized enrichment score; REGONIVO, regorafenib plus nivolumab; TASNIVO, TAS116 plus nivolumab; TGF, transforming growth factor.
CMS4 were detected in 0, 7 (35%), 0, and 13 (65%) cases in the REGONIVO trial and 4 (19%), 3 (14%), 2 (10%), and 12 (57%) cases in the TASNIVO trial, respectively. In the REGONIVO trial, CMS4 was significantly associated with patient response compared with the other CMS subtypes ($p=0.035$), but CMS4 was not associated with patient response in the TASNIVO trial (online supplemental table S2). Among 13 patients with CMS4 in the REGONIVO trial, one had a CR, six had a PR, and three had SD lasting more than 6 months. Patients with CMS4 in the REGONIVO trial demonstrated a significantly longer PFS (median 12.3 months vs 4.2 months; HR 0.208 (95% CI 0.062 to 0.693); $p=0.006$) and a longer OS (median 25.3 months vs 19.2 months; HR 0.621 (95% CI 0.196 to 1.968); $p=0.4139$) than did those with other CMS subtypes, whereas those with CMS4 in the TASNIVO trial did not (figure 5). In addition, when considering only cases of non-liver metastasis, a significant improvement in PFS was observed in patients with CMS4 in the REGONIVO trial (median 15.0 months vs 4.1 months; HR 0.072 (95% CI 0.006 to 0.808); $p=0.006$), which was not observed in cases with liver metastasis (online supplemental figure S3).

Multiplex fluorescence immunohistochemistry

mIHC of FFPE specimens obtained prior to treatment was performed to compare the tumor immune cell infiltration of responders and non-responders in the REGONIVO and TASNIVO cohorts using image analysis software (figure 6A). In the REGONIVO trial, the density of CD8$^+$ T cells (CD3$^+$CD8$^+$), Treg cells (FOXP3$^+$CD3$^-$CD8$^-$), and M2 macrophages (CD206$^+$CD11b$^+$) in the intratumoral area was significantly higher in responders (n=13) than in non-responders (n=9) (figure 6B). In contrast, M2 macrophage density was significantly lower in the responders (n=6) than in the non-responders (n=15) (figure 6C) in the TASNIVO trial. Similar trends were observed when focusing on primary lesions (online supplemental figure S4). In the combined analysis of samples from both trials, higher infiltration of CD8$^+$ T cells was observed in the CMS4 subtype than in the CMS2 and CMS3 subtypes, and infiltration of Treg cells and M2 macrophages was also

Figure 5  Survival curves based on the CMS classification Kaplan-Meier plots the PFS (A) and OS (B) of patients in the regorafenib plus nivolumab trial with tumors classified as CMS4, or as other CMS subtypes. PFS (C) and OS (D) of patients in the TAS116 plus nivolumab trial with tumors classified as CMS4 or as other CMS subtypes. CMS, consensus molecular subtypes; OS, overall survival PFS, progression-free survival.
observed; however the differences were not statistically significant (online supplemental figure S5). One patient harboring POLE mutations demonstrated a higher-than-average infiltration of CD8+ T cells with lower infiltration of Treg cells and M2 macrophages. Furthermore, in line with transcriptome analysis, responders (n=7) presented higher PDGFRα expression than non-responders (n=8) in the REGONIVO trial (online supplemental figure S6A, B).

We also evaluated the association between PD-L1 CPS, which is commonly associated with ICI response, and the proportion of responders, however, there was no significant difference (figure 3 and online supplemental table S2).

**Figure 6**  Multiplex immunohistochemistry analysis of the tumor immune microenvironment. Representative multiplex IHC images of samples from responders and non-responders in the REGONIVO and TASNIVO trials (A). Comparative analysis of tumor-infiltrating immune cells, CD8+ T cells (CD3+CD8+), Treg cells (FOXP3+CD3−CD8−), and M2 macrophages (CD206+CD11b+), was performed by multiplex IHC and HALO image analysis software between responders and non-responders in the REGONIVO (B) and TASNIVO (C) trials. IHC, immunohistochemistry; REGONIVO, regorafenib plus nivolumab; TASNIVO, TAS116 plus nivolumab.
DISCUSSION
To identify predictors of response to ICI combinations in patients with MSS or pMMR CRC, we conducted comprehensive biomarker analyses using WES, RNA-seq, and miHIC on the two investigator-initiated trials combining nivolumab with drugs expected to activate the immune response. We identified molecular features associated with the response to ICI combinations, particularly those related to tumor microenvironmental factors including EMT pathways and CMS4. To our knowledge, this is the first report to establish a multimolecular landscape of the response to ICI combinations in MSS or pMMR CRC.

We found that POLE mutations were significantly associated with response in the TASNIVO trial and that no specific gene mutations were associated with response in the REGONIVO trial. POLE mutations have been reported to be associated with a hypermutation phenotype and response to anti-PD-1 monotherapy in CRC.\textsuperscript{21–24} In this study, one patient in the TASNIVO trial with POLE mutations had an extremely high TMB and high CD8\textsuperscript{+} T-cell infiltration and low Treg cell and M2 macrophage infiltration. Thus, it is highly likely that the response achieved in the two cases with POLE mutations identified in the TASNIVO trial was primarily driven by nivolumab. However, aside from POLE mutation, no genomic features, including TMB, were identified as predictive markers for the response to ICI combinations in MSS or pMMR CRC in each study.

With respect to the tumor microenvironment, transcriptome analysis of samples from patients in the REGONIVO trial revealed upregulation of the EMT pathway and genes related to CAFs in responders and upregulation of the MYC pathway in non-responders. Furthermore, miHIC results revealed that the density of CD8\textsuperscript{+} T cells, Treg cells, and M2 macrophages was significantly higher in responders, which was comparable to the findings presented in a previous report.\textsuperscript{25}

Interestingly, patients with CMS4 in the REGONIVO trial were associated with better clinical outcomes, which was not observed in the TASNIVO trial. CMS4 is characterized by “mesenchymal” features, such as upregulation of the EMT and transforming growth factor (TGF)-β signaling pathways along with the high expression of genes associated with angiogenesis or extracellular matrix remodeling resulting in a high presence of CAFs,\textsuperscript{19,26} which are known to be associated with treatment resistance. Although the tumor immune microenvironment of CMS4 is considered “immune inflamed” with the presence of a higher number of infiltrating CD8\textsuperscript{+} T cells compared with CMS2 or CMS3,\textsuperscript{19,27} immune suppressive cells, such as Treg cells and M2 macrophages, which are involved in inhibiting cytotoxic T cells and suppressing the immune response,\textsuperscript{27} also infiltrated this subtype. It has been reported that Treg cells are recruited via CD70 expressed on CAFs in CRC, and accumulate due to CCL28 in the hypoxic environment caused by abnormal angiogenesis.\textsuperscript{28,29} In the tumor microenvironment regorafenib leads to a decrease in Treg cells with the inhibition of CAF proliferation inducing apoptosis and potent antiangiogenic effects, which are expected to improve the hypoxic environment.\textsuperscript{30,31} It has also been reported that regorafenib inhibits TAM infiltration and M2 macrophage activation by blocking the TIE2 pathway, thereby promoting a persistent M1 phenotype.\textsuperscript{30,32,33} Indeed, in preclinical models, regorafenib modified the tumor immune microenvironments decreasing the infiltration of CAFs, Treg cells and M2 macrophages, thus restoring the antitumor activity of PD-1 inhibitors.\textsuperscript{34,35} Additionally, it has been reported that PDGFRα, PDGFRβ, and KIT, which are targets of regorafenib, are highly expressed in CMS4 CRC and have been proposed as therapeutic targets.\textsuperscript{36,37} Indeed, our study found that PDGFRα was highly expressed in the REGONIVO trial responders, which is consistent with these reports. These findings suggest that combining regorafenib and PD-1 inhibitors could be effective for some CRC, specifically for the CMS4 subtype, in which infiltrating CD8\textsuperscript{+} T cells are suppressed by immunosuppressive cells.

In contrast, CMS2, which accounted for more than half of the non-responders in the REGONIVO trial, is associated with upregulation of the MYC and WNT signaling pathways, low levels of tumor-infiltrating immune cells, and poor intertumoral immune cell activation.\textsuperscript{19,26,27,38} Consistent with our finding that there was no correlation between CMS4 and REGONIVO response or a favorable clinical outcome in patients with liver metastases, preclinical models have indicated that the presence of liver metastases induces apoptosis in antigen-specific activated T cells, resulting in a systemic immunological desert.\textsuperscript{39} The development of further ICI combinations may be needed to address certain molecular subtypes and immune microenvironment phenotypes.

Transcriptome analysis showed upregulation of the G2M checkpoint pathway in the TASNIVO trial responders. WEE1, a client protein of HSP90, regulates the G2/M transition in the cell cycle by phosphorylating cyclin-dependent kinase 1.\textsuperscript{40,41} The AKT pathway and the MAP kinase cascade may be inhibited by HSP90 blockade.\textsuperscript{42,43} Thus, the HSP90 inhibitor TAS-116 may exert antitumor activity in tumors with elevated G2M checkpoint-related genes or high expression of AKT1 and HRAS in the TASNIVO trial. We previously reported that TAS-116 enhanced the antitumor activity of PD-1 inhibitors by reducing Treg cells in vitro and in vivo.\textsuperscript{44} However, in the present study, the significant infiltration of M2 macrophages in non-responders suggests that even if Treg cells were eliminated, the immune suppression by M2 macrophages could not be overcome by the HSP90 inhibitor.

This study has several limitations. The primary limitation is that this study was conducted with a limited sample size of patients from early clinical trials, and not all patient data were included in the biomarker analyses due to inconsistent sample availability. For example, only CMS2 and CMS4 were observed among patients in the REGONIVO
trial, probably due to the small number of included patients. Therefore, the presented results should be interpreted as preliminary, and further studies are warranted to validate these findings. Furthermore, because our analysis was performed using pretreatment samples only, a future comparative analysis of pretreatment and post-treatment samples would potentially strengthen our findings regarding the tumor microenvironment.43

In conclusion, we identified molecular features, particularly those related to tumor microenvironmental factors, that were associated with the response to REGONIVO and TASNIVO. Of note, CMS classification may correlate with those features, which were associated with the response to REGONIVO and TASNIVO. Of note, CMS classification may correlate with those features, which were associated with the response to REGONIVO and TASNIVO. Of note, CMS classification may correlate with those features, which were associated with the response to REGONIVO and TASNIVO. Of note, CMS classification may correlate with those features, which were associated with the response to REGONIVO and TASNIVO.

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**Correction notice** This article has been corrected since it was first published. The footnote has been added that ST, YT and Y-TL contributed equally.

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**Contributors** ST, YT, SKoy, KS, and AK designed the study. ST, YT, Y-TL, SKoy, TU, SKoj, MW, NS, MK, and TKu processed the experimental data and performed the analysis. SF, HH, YN, YK, DK, TKo, HB, and SM performed sample preparation. TY, HK, HN, IM, IE, KS, and AK performed conceptualization and supervised the project. ST, YT, Y-TL, and AK wrote the manuscript with input from all coauthors. KS and AK are guarantors of this study. All authors have read, reviewed, and edited the manuscript and approved the final version for submission.

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**Competing interests** ST, YT, and Y-TL have nothing to disclose. SKoy has received research funding from Otsuka Pharmaceutical, Shinogi Pharmaceutical and Chugai Pharmaceutical outside the submitted work. SF has nothing to disclose. HH has received honoraria from Bayer, Bristol-Myers Squibb, Chugai, Daiichi-Sankyo, Kyowa Hakko Kirin, Lilly, Merck Biopharma, MSD, Ono, Taiho, Takeda, and Yakult; fees for consulting or advisory roles for Bristol-Myers Squibb, Boehringer Ingelheim and Daiichi-Sankyo; and research grants from ALX Oncology, Amgen, Astellas, AstraZeneca, Bayer, Beigene, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai, Daiichi-Sankyo, Eisai, Janssen, Merck Biopharma, MSD, Ono Pharmaceutical, and Taiho Pharmaceutical outside the submitted work. YN has received honoraria from Chugai, Merck, and Guardant Health AIMEA and research grants from Taiho, Chugai, Guardant Health, Genomedica, Daiichi-Sankyo, Seagen, and Roche Diagnostics outside the submitted work. YK has nothing to disclose. DK has received personal fees for advisory roles from Takeda and Eisai; honoraria from MSD, Pfizer, Chugai, Merck biopharma, Lilly, Symex, Nipponkayaku, Bristol-Myers Squibb, Ono Pharmaceutical, Taiho Pharmaceutical, Takeda, Eisai, and Daiichi-Sankyo; and research funding from Ono Pharmaceutical, MSD, Novartis, Sanofi, Janssen, Pierre Fabre Medicament, Isosol Medical AB, Hutchison MediPharma Limited, and Nippon S器vier outside the submitted work. TKo has received honoraria from MSD, Bristol-Myers Squibb and Ono Pharmaceutical; personal fees for advisory roles from Boehringer Ingelheim, Kyowa Kirin and Taiho Pharmaceutical; and research funding from AstraZeneca, Beigene, MSD, Amgen, Chugai Pharmaceutical, Taiho Pharmaceutical, Shinogi Pharma, and Amgen Astellas BioPharma outside the submitted work. HB has received honoraria from Ono Pharmaceutical, Taiho Pharmaceutical, and Eli Lilly Japan and research funding from Ono Pharmaceutical outside the submitted work. SM, TU, SKoj, MW, NS, and MK have nothing to disclose. TKu has received honoraria from AstraZeneca, Astellas Pharma, Ono Pharmaceutical, Bristol-Myers Squibb Japan, Daiichi-Sankyo, and Roche Diagnostics; personal fees for consulting or advisory roles from Astellas Pharma; and research funding from Daiichi-Sankyo and Roche Diagnostics outside the submitted work. TY has received research grants from Taiho, Ono, Chugai, Amgen, MSD, Daiichi-Sankyo, Eisai, FALCO biosystems, Genomedia, Molecular Health, Nippon Boehringer Ingelheim, Pfizer, Roche Diagnostics, Symex, and Sanofi outside the submitted work and honoraria from Bayer, Chugai, Merck Biopharma, MSD, Ono, and Takeda. HN has received research funding and honoraria from Ono Pharmaceutical, MSD, Bristol-Myers Squibb, and Chugai Pharmaceutical and research funding from Taiho Pharmaceutical, Daiichi-Sankyo, Kyowa Kirin, Zenyaku Kogyo, Oncoylis BioPharma, Debiopharma, Asahi-Kasei, Symex, Fujifilm, SRL, Astellas Pharmaceutical, Sumitomo Dainippon Pharma, and BD Japan outside the submitted work. HM has nothing to disclose. IE has received research grants from Taiho Pharmaceutical and Ono Pharmaceutical outside the submitted work. AK has received personal fees for advisory roles from Roche and Lilly; honoraria from Roche, Merck Biopharma, Lilly, Bristol-Myers Squibb, Ono Pharmaceutical, Taiho Pharmaceutical, and Daiichi-Sankyo; and research funding from Ono Pharmaceutical, MSD, Sanofi, AstraZeneca, and Eisai outside the submitted work.

**Patient consent for publication** Not applicable.

**Ethics approval** The study was approved by the National Cancer Center Hospital East Certified Review Board (ID: 2022-041). Participants gave informed consent to participate in the study before taking part.

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**Data availability statement** Data are available upon reasonable request. Data access will be granted upon request to the corresponding author.

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**REFERENCES**

Microsatellite Stable Metastatic Colorectal Cancer Harboring a G"
SUPPLEMENTAL MATERIAL

Table S1. Sample information ..................................................... 2
Figure S1. Box plots comparing TMB in responders and nonresponders. .............. 4
Figure S2. Gene expression comparisons in REGONIVO and TASNIVO trials responders and nonresponders .............................................................. 5
Figure S3. Survival curves based on the CMS classification in patients with and without liver metastasis ................................................................. 6
Figure S4. Box plots comparing tumor-infiltrating immune cells ......................................................... 8
Figure S5. Box plots comparing tumor-infiltrating immune cells by CMS subtype ........ 9
Figure S6. Multiplex immunohistochemistry analysis of the tumor immune microenvironment  ................................................................. 10
## Table S1. Sample information

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Table S2. Number of responders and nonresponders per gene mutation, TMB, PD-L1 CPS, and CMS status

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Abbreviations: CPS, combined positive score; TMB, tumor mutational burden; CMS, consensus molecular subtypes
Figure S1. Box plots comparing TMB in responders and nonresponders

Box and whisker plots comparing TMB values in responders and nonresponders in each trial. POLE cases were excluded.

Abbreviation: TMB, tumor mutation burden
Figure S2. Gene expression comparisons in REGONIVO and TASNIVO trials responders and nonresponders.

The expression levels of the genes significantly upregulated in responders in the REGONIVO (A) and TASNIVO (C) trials (as indicated) were compared with the expression levels in nonresponders. Figure S2B shows the upregulation of genes related to cancer-associated fibroblasts in responders in the REGONIVO trial.

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B

MISHRA_CARCINOMA_ASSOCIATED_FIBROBLAST_UP

ES: 0.67, NES: 2.07
Nominal P-value < 0.01,
FDR Q value < 0.01

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Figure S3. Survival curves based on the CMS classification in patients with and without liver metastasis

Kaplan–Meier plots comparing PFS and OS between CMS4 and non-CMS4 patients with (A) and without (B) liver metastasis. PFS (A) and OS (B) in patients the REGONIVO trial with and without liver metastasis, respectively, and PFS (C) and OS (D) in the TASNIVO trial with and without liver metastasis, respectively. Abbreviations: PFS, progression-free survival; CMS, consensus molecular subtype; CMS4, consensus molecular subtype 4; OS, overall survival.
Figure S4. Box plots comparing tumor-infiltrating immune cells

Comparative analysis of tumor-infiltrating immune cells in the primary lesions of REGONIVO (A) and TASNIVO (B) trial responders and nonresponders using multiplex IHC.

A

CD3⁺CD8⁺  FOXP3⁺CD3⁺CD8⁻  CD206⁺CD11b⁺

P=0.0299  P=0.0594  P=0.0091

B

CD3⁺CD8⁺  FOXP3⁺CD3⁺CD8⁻  CD206⁺CD11b⁺

P=0.4537  P=0.1228  P=0.0358
Figure S5. Box plots comparing tumor-infiltrating immune cells by CMS subtype

Combined analysis of samples from the REGONIVO and TASNIVO trials comparing tumor-infiltrating immune cells between CMS subtypes.

- **CD3⁺ CD8⁺**: p = 0.1016
- **FOXP3⁺ CD3⁺ CD8⁻**: p = 0.5595
- **CD206⁺ CD11b⁺**: p = 0.2252
Figure S6. Multiplex immunohistochemistry analysis of the tumor immune microenvironment

Representative multiplex IHC images (A) and quantitative analysis of PDGFRα expression of specimens from responders and nonresponders in the REGONIVO trials (B).