Molecular and functional profiling unravels targetable vulnerabilities in colorectal cancer

Efstathios-Iason Vlachavas[1,#,*], Konstantinos Voutetakis[2,#], Vivian Kosmidou[2], Spyridon Tsikalakis[1], Spyridon Roditis[3,4], Konstantinos Pateas[3], Ryangguk Kim[5], Kymberleigh Pagel[6], Stephan Wolf[7], Gregor Warsow[8], Antonia Dimitrakopoulou-Strauss[9], Georgios N Zografos[3], Alexander Pintzas[2], Johannes Betge[10,11,12], Olga Papadodima[2,*], and Stefan Wiemann[1,*]

(1) Division of Molecular Genome Analysis, German Cancer Research Center, Heidelberg, Germany

(2) Institute of Chemical Biology, National Hellenic Research Foundation, Athens, Greece

(3) 3rd Surgical Department G.Gennimatas Hospital, Athens, Greece

(4) University Hospital of North Midlands, Surgical Department, Stoke-on-Trent, UK

(5) Oak Bioinformatics, LLC, Fairfax, VA, USA

(6) SpinSys-Diné, Falls Church, VA, USA

(7) High-throughput Sequencing Core Facility, German Cancer Research Center, Heidelberg, Germany

(8) Omics IT and Data Management Core Facility, German Cancer Research Center, Heidelberg, Germany

(9) Clinical Cooperation Unit Nuclear Medicine, German Cancer Research Center, Heidelberg, Germany

(10) Junior Clinical Cooperation Unit Translational Gastrointestinal Oncology and Preclinical Models, German Cancer Research Center (DKFZ), Heidelberg, Germany

24   (11) Department of Medicine II, University Medical Center Mannheim, Medical Faculty

25   Mannheim, Heidelberg University, Mannheim, Germany

26   (12) DKFZ-Hector Cancer Institute at University Medical Center Mannheim, Germany

27   # equal contribution

28   * corresponding authors

29 Abstract

While colorectal cancer (CRC) patients with microsatellite instability (MSI) respond well to

immunotherapy those with microsatellite stable (MSS) tumors rely on conventional chemotherapy,

often with poor outcomes. Both types frequently carry mutations in *KRAS* or *BRAF* proto-oncogenes,

rendering them more resistant to treatment. New therapeutic biomarkers and treatments remain a

clinical need, especially for MSS tumors. We performed whole exome and RNA-Sequencing from 28

tumors of the Athens Comprehensive Cancer Center CRC cohort, and molecularly characterized

colorectal cancer patients based on their MSI status, SNVs/CNAs, and pathway/transcription factor

activities at the individual patient level. Variants were classified using a new computational score for

integrative cancer variant annotation and prioritization. Complementing this molecular data with

public multi-omics datasets, we identified activation of transforming growth factor beta (TGFβ)

signaling to be stronger activated in the MSS patients whereas JAK-STAT and MAPK molecular

cascades were activated specifically in MSI. We unraveled mechanisms consistently perturbed in the

transcriptional and mutational circuits and identified RUNX transcription factors as putative novel

targets. Assessing the immunogenicity of CRC tumors in the context of RAS/RAF mutations and

MSI/MSS status revealed a critical impact *KRAS* mutations have on immunogenicity particularly in the

MSS patient subgroup, with implications for diagnosis and treatment.

46 Introduction

Colorectal cancer (CRC) is a highly heterogeneous disease associated with different molecular

background and clinical management. Improved screening strategies, surgical techniques and

advanced treatment protocols have led to better clinical outcomes of CRC patients in the last

decades [1, 2]. The recent development of covalent inhibitors specifically targeting the KRAS G12C

variant have indicated significant progress in targeting even previously 'undruggable' onco-proteins [3].

Despite these advances, CRC has remained the second and third leading cause of cancer-related

death in the US and worldwide, respectively [4, 5]. Furthermore, recent epidemiological data has

indicated a concerning trend of increased CRC incidence particularly in younger individuals below the

age of 55, coupled with more advanced disease stages [4]. The response to treatment is highly variable

and not durable in the majority of cases. This dismal clinical perspective is impacted by various

factors, including tissue tropism effects that allele-specific *RAS* mutants have in combination with

other actionable alterations [6], clonal heterogeneity [7], phenotypic plasticity [8], location of tumor [9], and

the tumor microenvironment [10]. Collectively, these findings highlight the need for novel therapies

focused specifically on the personalized treatment of CRC. The advent of next-generation sequencing

has led to substantial progress in molecular characterization of the disease. Alterations in a number

of genes with critical roles in CRC have been identified, such as in *APC, BRAF, EGFR, NRAS, KRAS* and

PIK3CA [11, 12]. The resulting aberrant activation of signaling pathways may be exploited in precision

therapies. Recent accomplishments include combinatorial strategies targeting co-activated pathways

in various types of unresectable CRC such as BRAF V600E mutated [1] and *HER2*-positive carcinomas [2].

The microsatellite instability (MSI) status is among the most informative parameters determining

treatment decision towards personalized medicine in CRC [13], and in other tumor entities [14]. According

to the MSI status, CRC is categorized into three types: high microsatellite instable (MSI-H), low

microsatellite instable (MSI-L) and microsatellite stabile (MSS) [13]. MSI-L and MSS tumors are

commonly grouped into one type [15]. Accordingly, we use MSS for MSI-L/MSS and MSI for MSI-H

tumors throughout. MSI-status accounts for ~15% of CRC cases and is associated with sporadic or

hereditary disease etiologies. While the former is associated with *BRAF* mutations [16] and *MLH1*

promoter methylation [17], the latter has been associated with mutations mostly in mismatch repair

genes *MLH1*, *MSH2*, *MSH6* as well as *PMS2* and is classified as Lynch syndrome [18]. MSI tumors have a

high mutational burden, resulting in the expression of neo-antigens that may be recognized by the

immune system. Differences in the immune microenvironment thus distinguish MSI and MSS CRCs,

and have immediate therapeutic impact: MSI CRC tumors mostly respond to immunotherapy, which

has revolutionized clinical practice and outcome of respective patients [19].

In contrast, MSS CRC patients commonly do not benefit from immunotherapies and have mostly

limited targeted treatment options, wherein the most informative molecular distinction involves the

RAS mutation status [20]. Despite the unmet therapeutic demand particularly for MSS patients, only

few studies have investigated the multi-layered differences in MSI/MSS tumors, their downstream

signaling effects, and their interrelation with *RAS/RAF* mutations in regard to immunogenicity and

pathway activation [21, 22]. Hence, by leveraging a prospective patient cohort of the Athens

Comprehensive Cancer Center (ACCC - https://www.accc.gr/index.html) and two public cancer

genomics datasets, we aspired to elucidate the molecular complexity of microsatellite (in)stability

status in CRC and to uncover biological mechanisms that might be exploited to enhance therapeutic

options particularly for patients with the MSS type.

Results

The mutational landscapes of MSS and MSI CRCs

To elucidate the landscape of somatic mutations in CRC, whole exome sequencing (WES) as well as

RNA sequencing (RNAseq) was performed in cancerous as well as in adjacent normal tissues from 28

patients of a prospective patient cohort of the Athens Comprehensive Cancer Center (ACCC)

(Supplementary Table 1). Since microsatellite instability is one of the major clinical factors

determining therapeutic management and prognosis of CRC patients, we first wanted to infer the

microsatellite stability status of every patient using RNAseq data. To this end, we initially validated

the robustness of PreMSIm [23] and predicted the MSI status in 434 patients of the TCGA PanCan

COAD/READ [11] as well as in 79 patients of the CPTAC [24] cohorts. PreMSIm estimates were highly

concordant with MSI-MANTIS predictions from TCGA (concordance rate: 0.988, Supplementary Table

2), and with the annotated MSI-status of patients in CPTAC (concordance rate: 0.987, Supplementary

Table 3). We then characterized the ACCC CRC samples applying PreMSIm and inferred five tumors as

MSI and 23 as MSS (Supplementary Table 4).

The exomes of ACCC CRC samples were sequenced at an average coverage of >300x (range: 195 to

375). In total, 25,913 unique protein-altering somatic variants were identified in 11,492 genes,

including 21,917 missense, 1495 stop_gained, 423 splice site, 1871 frameshift and 162 inframe InDel

mutations (Supplementary Table 5). The number of somatic variants varied greatly among ACCC

patients as the tumor mutational burden (TMB) ranged from 0.028 to 375.994 protein-altering

mutations per Megabase (Supplementary Table 4). The median was 108 nonsynonymous SNVs in

MSS tumors while that in MSI tumors was 1257. Five patients carried hot-spot mutations in the G12

codon of KRAS [G12D (n=3), G12V (n=1), G12S (n=1)], another five patients carried hot-spot

mutations in BRAF [V600E (n=4), K601E (n=1)], while concomitant mutations in KRAS [A146V] and

BRAF [D594N] were observed in one ACCC-patient. Another patient carried an HRAS [A146T]

mutation co-occurring with two non-hotspot mutations in BRAF [L505F and L577I]. The HRAS [A146T]

substitution has been found as a germline variant in Costello's syndrome [25] and as a somatic

mutation in a few cases of melanoma and other skin cancers (https://www.cbioportal.org, GENIE

Cohort v14.1-public, accessed on 2023/12/04). BRAF [L505F] is a class II mutation [26] and has been

shown to confer a RAS-independent constitutively activated state in the kinase domain [27]. This and

BRAF [L505H] have been reported in a small number of tumor entities including melanoma, prostate

and pancreatic cancer in cbioportal. BRAF [L505H] was shown to be activating mutation conferring

resistance to vemurafenib [28], and is characterized as oncogenic in OncoKB [29]

(https://www.oncokb.org/gene/, accessed 2023/12/13). The BRAF [L577I] mutation has been

reported in cutaneous melanoma and is characterized as variant with 'Unknown Oncogenic Effect' in

OncoKB.

In order to prioritize somatic mutations according to their putative oncogenicity, we next applied our

SVRACAS variant scoring pipeline (https://github.com/Jasonmbg/Simple.-Variant-Ranking-

Annotation-CAncer-Score, accessed 2022/10/24) to the 25,913 variants, leaving 4307 prioritized

variants (score ≥0.5) in 2969 genes (Supplementary Table 5). Amongst these, 54 matched with 72

CRC driver genes annotated IntOgen [30] (Fig. 1). The top frequently mutated genes in the ACCC tumors

were *APC* and *TP53* (Fig 1) and the presence of mostly loss-of-function mutations was in line with

previous observations [11, 31]. Next, we revealed differences in the 54 mutated genes that had matched

the IntOgen set, between the MSI and MSS CRC tumors of the ACCC cohort regarding somatic SNVs,

InDels and copy number alterations (CNAs). On the one hand, MSS tumors exhibited a higher

percentage of CNAs: Deletions of *SMAD2*, *SMAD4* and *MAP2K4*, and gains in *RNF6*, *BRCA2*, *NBEA* (chr

13q12-13) as well as in *GNAS* (chr 20q13.32) were only observed in MSS tumors. On the other hand,

frameshift and missense variants were more frequent (*RNF43*, *ACVR2A*, *TGFBR2*) or exclusive (*BCL9L*,

PTPRC) in the MSI cluster.

Mutational analysis implementing the COSMIC SBS signatures [32] revealed distinct patterns between

the MSI and MSS tumors (Supplementary Fig. 1). The majority of the MSI tumors were characterized

by SBS6, SBS26, and SBS20 signatures, all related to defective mismatch repair and, thus,

microsatellite instability. One sample (ACCC_CRC_15) was further characterized by signatures 10a

and 10b, which are related with mutations in *POLE*. Mutations in *POLE* are associated with a very

high TMB, which was indeed observed in this patient (375.994 mutations/Mb). On the contrary, MSS

samples were mainly enriched in the SBS1 and SBS5 signatures, which are related to age, stem-cell

division and "clock-like" processes, and are in line with a general low TMB in MSS tumors. However,

the DNA mismatch repair-associated signatures SBS6 and SBS15 were evident also in two MSS cases

(ACCC_CRC_05 & ACCC_CRC_06, respectively), possibly related to their high TMB. Patients

ACCC_CRC_07 and ACCC_CRC_18 were enriched with the unknown etiology signatures SBS93 and

SBS94, which have been identified in gastric and colorectal samples, respectively. Taken together,

these findings are in line with previous studies recapitulating the mutational landscapes of CRC,

where microsatellite instability is associated with a low frequency of CNAs and a high TMB [33, 34].

Mapping mutational landscapes to functional networks

To extend the analysis of mutational profiles in MSS and MSI CRC tumors, we utilized the public

datasets of the TCGA PanCan COAD/READ [11] and the CPTAC [24] cohorts. Complete datasets were

available for 434 patients (392 MSS, 42 MSI) from TCGA (Supplementary Table 2), and 79 patients (65

MSS, 14 MSI) from CPTAC (Supplementary Table 3). Highly scoring variants from SVRACAS annotation

(score ≥0.5) were identified in 7287 and 2588 genes in the TCGA and CPTAC datasets, respectively

(Supplementary Tables 6 and 7). Of those, only three genes had higher mutation frequencies in MSS

compared to MSI tumors: *APC* and *KRAS* genes were found more frequently mutated in MSS in both

public datasets, whereas mutations in *TP53* were significantly enriched only in the TCGA PanCancer

dataset. In contrast, 564 (TCGA) and 80 (CPTAC) genes had significantly higher mutation frequencies

in MSI compared to MSS tumors, and 42 genes were shared in both datasets. To uncover molecular

networks possibly related to microsatellite instability, we then performed functional enrichment

analysis by mapping the genes differentially mutated (MSS vs. MSI) in TCGA and CPTAC to Reactome

[35]. Eleven pathways were significantly enriched in MSI vs. MSS tumors in both, TCGA and CPTAC.

These signaling pathways were related to chromatin modification and organization, growth factor,

second messenger as well as Wnt signaling, and to RUNX1 transcriptional regulation (Fig. 2). In line

with only three genes having higher mutational frequencies in MSS vs. MSI, no Reactome pathway

was enriched in MSS tumors.

Next, we mapped the 2969 mutated genes from the ACCC CRC dataset to the 11 enriched Reactome

pathways. This identified 60 genes that were mutated with a higher frequency in ACCC MSI tumors

and in at least one of the two other CRC datasets. Functional and physical associations were

investigated by mapping these genes to the STRING database [36], identifying three protein interaction

networks with 60 genes and 154 edges (Supplementary Fig. 2). The first network comprised of genes

in angiogenesis as well as ERBB2, PI3K/AKT and PDGFR signaling. Network 2 was enriched in histone

modifications, NOTCH1, TCF and Wnt/β-Catenin signaling, while network 3 was characterized by

RUNX1 signaling, regulation of nucleotide-excision repair, double-strand break repair, and chromatin

remodeling. The Reactome and STRING network topologies mirrored prior knowledge of MSI CRC

thus further validating the ACCC CRC cohort and the classification of patients into MSS and MSI

subtypes [11, 12].

Molecular pathway and transcription factor activities are distinct in MSS vs. MSI mutated CRCs

Aiming to estimate the activity of cancer-relevant signaling pathways in MSS and MSI groups of the

three CRC datasets (TCGA PanCan COAD/READ [11], CPTAC [24] and ACCC CRC), we next estimated the

activity of 14 signaling pathways based on transcriptomic data using the PROGENy computational

pipeline [37]. The activity of only the TGFβ signaling pathway was significantly higher in MSS than MSI

tumors in all three cohorts (Fig. 3A). This is in line with a recent report suggesting that TGFβ signaling

in MSS tumors stimulates cancer associated fibroblasts and establishment of an immune-excluded

tumor microenvironment [38]. In contrast, six pathways were stronger activated in MSI tumors in all

three cohorts (Fig. 3A). JAK-STAT signaling was the most activated pathway in MSI and has been

implicated with positive immunotherapy outcomes [39]. Then, kinase and pathway activity analysis was

performed using the phospho-proteomics measurements from the CPTAC cohort. This suggested the

cAMP-dependent protein kinase and TGFβ signaling pathways as more active in MSS tumors

compared to MSI (Supplementary Fig. 3A), confirming the PROGENy results for TGFβ. Activation of

Protein kinase A might be functionally related to gains and correlated expression of *GNAS* (r=0.45, p-

value < 0.05) found specifically in MSS tumors also in the CPTAC cohort (Supplementary Fig. 3C, D).

This pathway has been proposed as targetable driver mechanism in lung cancer [40]. In contrast,

several MAP kinases and PI3K signaling were significantly more activated in the MSI tumors

(Supplementary Fig. 3A, B). The latter findings confirm that MSI tumors exhibit a higher activation of

stress-response, inflammation/immune-modulation, and proliferation.

Next, we performed transcription factor (TF) activity analysis in MSS vs. MSI tumors and found 14 TFs

that had consistently different activity profiles in all three datasets. Six transcription factors including

RUNX2 (Runt-related transcription factor) were stronger activated in MSS CRCs compared to MSI in

all datasets (Fig. 3B). RUNX transcription factors are downstream effectors of TGFβ, thus matching

the results from pathway analysis. These TFs regulate basic cellular and developmental processes,

stem cell biology, and tumorigenesis [41]. In colorectal cancer, RUNX2 influences MAP kinase signaling

via regulation of multiple RTKs, and its absence, can lead to resistance against MEK inhibitors along

with its cofactor CBFB [42]. MSI CRCs presented significantly higher activities for the MYC, STAT2,

HIF1A, TP53, SPI1, IRF1 and IRF2 transcription factors (Fig. 3B). Some of these have previously been

associated with CRC and potentially impact immunogenicity, proliferation, DNA repair mechanisms

and cellular stress responses [43]. Collectively, pathway and transcription factor activation-profiling

point at elevated inflammatory processes in MSI tumors while tumors of the MSS type appear to be

associated with stem-cell features as well as an immune-excluded microenvironment.

Transcriptomic stratification and immune deconvolution of ACCC-CRC samples

We then estimated altered pathway and TF activities at the individual patient level to achieve a

personalized view on the ACCC patients. To this end, we used the scaled gene-expression values of

every patient as input and focused on the seven molecular pathways and 14 TFs, which we had found

significantly altered in the three cohort-based comparison of MSS vs. MSI CRC tumors. The resulting

estimated pathway and TF activities grouped the ACCC CRC patients into three patient clusters (Fig.

4A). To gain further insights into the underlying mechanisms that characterize the identified clusters,

we performed single sample Gene Set Enrichment Analysis (ssGSEA) [44], immune deconvolution [45],

and inference of Consensus Molecular Subtypes (CMS) [46].

The first patient cluster (Cluster_ID 1, n=13 patients) mostly included MSS (n=12) and CMS2 and

CMS3 cancers (Fig. 4A). In line with MSS tumors in the TCGA and CPTAC (Fig. 3A), the tumors in

cluster 1 were characterized by a low immunogenic behavior with sparse activation of the JAK-STAT

and NFkB inflammatory pathways, no enrichment of immune related hallmarks, and flat immune

infiltration scores (Fig. 4B). These findings are in line with the poor response MSS patients mostly

have to immunotherapies. Patient ACCC_CRC_15, the only MSI sample in this cluster, also showed an

overall trend of under-activation in the majority of estimated pathway and TF activities, whereas the

EGFR signaling cascade and MYC TF were found significantly over-activated in this *POLE*-mutant

tumor. This tumor had been diagnosed as serrated mucinous adenocarcinoma, which might explain

the separate clustering from the other MSI tumors [47]. The second cluster (Cluster_ID 2, n=7)

contained only MSS and mostly CMS4 samples (n=6) which were characterized by an overall

significant activation of the TGFβ signaling pathway, expression of genes associated with epithelial to

mesenchymal transition (EMT) and angiogenesis as well as activation for the SPI1, RUNX2 and

PRDM14. Almost all samples in the second cluster exhibited low activity of E2F4 and MYC,

accompanied with reduced cell cycle activity and unfolded protein response (Fig. 4A, B). This suggests

that tumors in the second cluster are characterized by an overall anti-proliferative capacity and a

more invasive phenotype, which could be related to the molecular and clinical characteristics of the

mesenchymal subtype (CMS4) [48]. The third cluster (Cluster_ID 3, n=8) comprised four MSI and four

MSS samples. It was mainly composed of CMS1 and CMS2 tumors with the exception of sample

ACCC_CRC_21 that was predicted as CMS3. Tumors in cluster 3 were characterized by a relatively

stronger activation of inflammatory (JAK-STAT, NFkB) and mitogenic (EGFR and MAPK) signaling

pathways, compared to patients in the other clusters. Moreover, MYC and E2F4 seemed to be

strongly activated. Conversely, a group of TFs, mainly associated with chromatin remodeling

(PRDM14, ASCL1, RUNX2) and developmental processes (HNF4A, TCF4) displayed a reduced activity

level compared to the other two clusters. This was most evident in the MSI samples of the third

cluster, while MSS samples in this cluster showed a more variable activation pattern. Collectively,

patients forming the third cluster reflected a more immune-reactive and less aggressive molecular

profile.

The MSS tumors showed an overall higher degree of heterogeneity, which was associated with the

prevalence of *KRAS* and *BRAF* mutations. *KRAS*-mutated MSS samples showed a trend towards

having lower activation of immune response pathways, i.e. having significantly lower activities in JAK-

STAT and NFkB pathways relative to the RAS_RAF_GNAS_wt MSS samples in the second patient

cluster (Fig. 4A). This was corroborated by ssGSEA findings and immune deconvolution estimates (Fig.

4B), suggesting that *KRAS*-mutant MSS tumors in the first patient cluster were less immune reactive

compared to the MSS tumors in the other clusters. These findings are in line with the observed low

activity of IRF1 and IRF2, regulators of the interferon response [49], in the majority of the *KRAS*-mutant

MSS samples of the first cluster (Fig. 4A). This is consistent with previous studies, which have

identified a mutant *KRAS-IRF2* molecular cascade, promoting an immunosuppressive environment in

CRC and fostering immunotherapy resistance [50]. Conversely, the transcriptomic circuits of MSS CRC

tumors in the third patient cluster showed greater similarity to the respective circuits in the MSI

tumors within that cluster, compared to the MSS samples in the other clusters. In summary, while

the evaluated transcriptomic programs primarily differentiated MSS and MSI samples and

corresponded with the prevalence of identified CMS subtypes, they also exposed differentially

enriched patient-cluster and patient-specific gene expression networks.

From Oncogenic Mutational Landscapes and Transcriptomic Features Towards Personalized

Therapeutic Strategies

Having seen higher functional heterogeneity in MSS compared to MSI tumors and that only few

pathways seemed to be consistently stronger activated in MSS tumors, we hypothesized that

precision oncology approaches might be particularly useful for MSS CRC. Along these lines, we went

out to unravel actionable alterations for individual ACCC CRC patients and interrelate them with their

respective transcriptomic profiles. This should provide more holistic views on potential vulnerabilities

and frame them as comprehensive personalized cancer maps, placing our special emphasis on MSS

cases. For this purpose, the SVRACAS prioritized somatic variants in each patient were annotated

with clinical actionability-information from the OncoKB precision oncology database [29]. This analysis

prioritized 34 actionable variants in 20 genes, reflecting these variants as predictive biomarkers of

drug sensitivity or resistance to particular targeted therapies (Fig. 5A).

Next, we assigned the ACCC CRC patients to seven MASTER interventional baskets [51], based on the

mutational profiles the respective tumors presented with. The MSI-status and TMB were regarded

for assignments to the immune evasion (IE) basket. Seventeen patients of the ACCC cohort (~63%)

were stratified into at least one interventional basket (Fig. 5B). Tumors in patient cluster 1 were

mainly characterized by overrepresentation of the RAS-MEK-ERK (RME, 8 out of 9 patients), the PI3K-

AKT-mTOR (PAM, 3 out of 9 patients), and the immune evasion (IE, 3 out of 9 patients) baskets. An

interventional basket (i.e., RME) could be assigned to just one (ACCC_CRC_16) out of seven MSS

patients forming cluster 2, in line with the rather low TMB in the tumors forming this cluster. Cluster

3 was mostly represented by RME (5 out of 7 patients) and PAM as well as IE (4 out of 7 patients

each) baskets.

The five MSI- and two MSS-patients could qualify for treatment with immune checkpoint-inhibitors

(e.g., pembrolizumab), the MSS-patients in the light of the high TMB in the tumors (Fig. 5B). Two MSI

and another two MSS tumors carried BRAF V600E mutations and might benefit from treatment with

encorafenib and cetuximab [52]. Seven patients carried *KRAS* or *NRAS* mutations, rendering those

patients incompatible with EGFR-antibody therapy [53]. Inhibitors specific for the most common KRAS

variant G12D, which was present in three cases, are currently in preclinical and clinical development

(e.g. MRTX1133, NCT05737706). Tumors carrying druggable KRAS G12C mutations [3] were not present

in our cohort. No specific treatments were clinically available for the patients carrying other RAS

mutations, i.e., ACCC_CRC_13 (G12V) and ACCC_CRC_27 (G12S). However, also non-specific Pan-

KRAS inhibitors are in development [54], which might be applied once having proven efficacious.

Hence, molecular profiling identified potential treatment options for the majority of patients in the

cohort and more than one interventional basket was mapped to eight patients, suggesting a

combination of targetable driver mechanisms in the respective patients.

We finally went ahead with two patients of the MSS type to prove the utility of our approach

towards suggesting potential vulnerabilities for tailored therapeutic interventions. Patients

ACCC_CRC_05 and ACCC_CRC_06 had presented with adenocarcinomas of the CMS3 subtype and

had a right-sided location. Patient ACCC_CRC_05 (Fig. 5C) was characterized by an ERBB2 p.R678Q

mutation, which has a modest biological rationale for treatment with trastuzumab-deruxtecan [55]

(NCT evidence level m4). Furthermore, this patient carried an ATM p.F61Lfs*15 mutation, which has

been found in few, mostly colorectal tumors (CosmicID: COSV53735143, accessed 2023/12/14).

While preclinical evidence supports PARP inhibition [56] (NCT evidence level m3) this was recently

shown (NCT02693535) to be ineffective in *ATM*-mutated CRC [57] thus not giving it high priority. In

patient ACCC_CRC_05, we identified *PTEN* p.T319* and TSC2 p.R1451Afs.25 mutations, both of which

have biological justification and preclinical evidence [58] suggesting treatment with mTOR inhibitors

(NCT evidence level m3-m4). Tumor gene expression analysis for this patient demonstrated an over-

activation of PI3K/AKT/mTOR signaling. The data further suggested an under-activation in the EGFR

and JAK-STAT signaling pathways, which might indicate a predominantly PI3K/mTOR-driven cancer

phenotype for this patient, further supporting the rationale for mTOR inhibition.

MSS patient ACCC_CRC_06 carried several potentially druggable alterations (Fig. 5C). The BRAF

V600E mutation in this tumor could be targeted with Encorafenib and Cetuximab as an approved

combination for second line targeted therapy after chemotherapy [1]. BRCA1 p.K654Sfs*47 and BRCA2

p.T3085Qfs*19 mutations in this patient suggested PARP inhibition as a potential option. Tumor

transcriptomic analysis revealed an over-activation of the PI3K/AKT/mTOR and under-activation of

the DNA repair and JAK/STAT molecular circuits. A frameshift mutation in *TSC2* (p.N1017Tfs*13)

would suggest mTOR inhibition as therapeutic strategy [59]. However, in contrast to patient

ACCC_CRC_05, we noticed a trend towards activation of the MAPK signaling pathway in patient

ACCC_CRC_06, which might be associated with the presence of the BRAF V600E mutation. Thus, the

value of targeting mTOR in the context of an activating BRAF mutation seems questionable [60].

Patients ACCC_CRC_05 & _06 both presented with high TMB, which has been observed in MSS

tumors before [31], and mutational signatures associated with DNA mismatch repair (SBS6 and SBS15

in patients ACCC_CRC_05 and ACCC_CRC_06, respectively). While the TMB might indeed indicate

application of immunotherapies, the lack of mutations in *POLE* [61], and the modest stromal and

immune signatures in these tumors were not supportive of this (Fig. 4), suggesting immune

checkpoint-inhibition as an only hypothetical treatment option [62]. Yet, our analysis pointed at

rationale treatment options for these two as well as for most other patients in the ACCC CRC cohort.

Collectively, our study underlines the significant value of incorporating various molecular and

functional layers to support therapeutic decision-making.

Discussion

Here, we set out to characterize the molecular and activity landscapes MSS and MSI colorectal

cancer. By implementing an integrative computational workflow we analyzed tumors from 28

patients of a prospective CRC patient cohort of the Athens Comprehensive Cancer Center (ACCC), in

combination with two large public multi-omics datasets. There, we explored the molecular profiles of

MSS vs. MSI CRCs, their relation to *KRAS/BRAF* mutations, and pathway as well as transcription factor

activities in the context of personalized medicine. Firstly, we observed novel associations between

particular genes and their increased mutational frequencies specifically in MSI tumors. Mutations

were enriched in genes that are involved in molecular pathways mainly related to transcriptional

regulation cascades by RUNX1, chromatin modification mechanisms, and in signaling cascades that

are related to Reactome 'fusion mutants/proteins' (i.e., FGFR1/FLT3). These findings resonate with

research indicating the central role of RUNX1 in the malignant transformation process and metastasis

of CRC through mechanisms, like the Wnt/β-catenin signaling pathway and EMT [63, 64]. Fusion events

involving FGFR have been considered as therapeutic targets in various solid tumors [65]. In contrast,

MSS tumors demonstrated a pronounced enrichment in CNAs. Deletions were recurrently found in

SMAD2, *SMAD4*, and *MAP2K4*, which are pivotal in the TGFβ and JNK signaling pathways [66, 67],

respectively. Gains were observed in chromosomes 13q12-13 and 20q13.32, which have been

associated with early events in the development of colorectal cancer [68], also affecting *GNAS*. These

CNAs exclusively presented in MSS, suggesting a unique MSS genomic signature with implications in

MSS disease etiology and therapeutic targeting [69].

Secondly, our comprehensive multi-omics analysis indicated differentially regulated circuits at the

transcriptional level. Immune related biological mechanisms, such as JAK-STAT and $NF_\kappa B$ signaling

pathways, were found upregulated in MSI tumors, consistent with the higher 'immunogenicity' in the

context of microsatellite instability [70]. Conversely, the TGFβ pathway showed greater activation in

the MSS tumors and has been associated with epithelial-to-mesenchymal transition (EMT),

metastasis, and therapy resistance [71, 72]. We further observed that the synchronous presence of *KRAS*

mutations with microsatellite instability had a stronger effect on the immune profile of CRC tumors

than mutations in *BRAF*. Hence, we identified context-specific functional associations that are likely

related to the higher functional heterogeneity within MSS CRC tumors. Previous studies have

emphasized the intricate role *KRAS* mutations play in shaping the tumor microenvironment (TME) of

colorectal cancer. These mutations seem to foster an immunosuppressive environment, which is

marked by a diminished anti-tumor immune response [73].

Next, we identified transcription factors with differential activity between MSS and MSI CRC tumors.

MSI CRC tumors showed higher activities of MYC, STAT2, HIF1A, TP53, SPI1, IRF1, and IRF2. These

factors are instrumental in various cellular processes, such as cell growth, response to stress, oxygen

homeostasis, and immune modulation [43]. Hence, therapeutic regulation of TFs might be a promising

strategy for CRC therapy owing to their underlying role as key effectors of various signal transduction

and tumor-associated immune responses [74]. Distinct transcriptional profiles were observed in MSS

CRCs, indicating the heightened activity of RUNX2 upstream of TGFβ signaling [75]. Yan et al. revealed

that RUNX2 is critical for the maintenance of the stem cell-like properties of CRC cells as well as for

the promotion of CD44-induced EMT in CRC[76]. Collectively, from our integrative analysis we noticed

the significant deregulation of specific members of the RUNX TF family at DNA (*RUNX1*) or mRNA

(*RUNX2*) levels, supporting their emerging role as prognostic biomarkers in CRC [77].

Our comprehensive molecular tumor analysis thus corroborated prior findings related to the

phenotypic characteristics of CMS subtypes, however, also suggests that the microsatellite status is

not the only biomarker that could predict molecular and functional tumor characteristics. In our

integrative clustering analysis, one cluster grouped MSI with MSS patients together. This could be

explained, in part, with epigenetic reprogramming in tumor progression, playing a major role in the

clonal evolution and tumorigenesis of CRC [78]. Indeed, this aligns with a recent study proposing a

novel molecular classification of CRC tumors based on single-cell sequencing data [79]. This study

identified a subgroup of MSS samples (iCMS3_MSS), which exhibited more similarity with the

transcriptional and genomic levels of MSI CRC tumors (iCMS3_MSI), forming a novel epithelial

subtype [79]. This may indicate that the MSI status, even though clearly distinct at the genomic level,

may be viewed as a continuum through different states and sub-conditions at the transcriptomic

layer [80]. The potential therapeutic consequences of this, however, remain to be uncovered.

Finally, we sought to provide a personalization of molecular findings, as the identification of

targetable alterations is a primary focus of current clinical research, to facilitate personalized

treatment strategies. Effective therapeutic options exist for MSI CRCs employing immune checkpoint

inhibitors. In contrast, the management of MSS cancers still predominantly relies upon conventional

chemotherapies, as the most frequently encountered mutations in MSS CRC affect non-druggable

tumor suppressor genes, such as *APC* and *TP53*. Indeed, the prevalence of well-established

targetable mutations with clinical evidence supporting therapeutic interventions is exceedingly low in

CRC. These primarily encompass BRAF V600E mutations, which we observed also in our cohort and

have proven more difficult to target in CRC compared, for example, to melanoma [52]. Clinical

treatment options are often limited even when mutations in potentially druggable drivers have been

identified [81]. Hence, the integration of expression data might prove beneficial in clarifying

therapeutic decisions for patients with uncertain mutation-driven treatment options, which we

illustrated with patients ACCC_CRC_05 and ACCC_CRC_06. Our integrated analysis suggested

treatment options for these patients (PARP/mTOR inhibition) that were unknown when the patients

were indeed in clinical treatment.

The added value of integrating molecular modalities to foster precision medicine was initially

introduced with the WINTHER clinical study [82] (NCT01856296). WINTHER was the first trial to

describe the combination of matched genomic and transcriptomic information in metastatic tumors,

showcased the significance of integrated molecular profiling into improved therapeutic

recommendations, and expanded the portfolio of putative therapeutic interventions [82]. Likewise, the

more recent MASTER/NCT clinical study has illustrated that the integration of WES/WGS with RNA

sequencing can result in a clinical benefit and boost patient treatment stratification in patients with

advanced cancer of rare cancer entities [51]. Our findings support these concepts, while we placed our

focus on MSS and MSI CRC, in the context of the mutation status in the *KRAS* and *BRAF* genes. Our

study initially suffered from the relatively small sample size of the retrospective ACCC cohort, which

impeded direct generalization of inferential statistics and related biological comparisons. To cope

with this limitation, we took advantage of public data sets of the TCGA and CPTAC cohorts,

constructed an integrative workflow, and comprehensively profiled the sequencing data from the

ACCC and the public multi-omics cancer datasets. Collectively, this allowed us to perform a patient-

centric analysis capitalizing on the available molecular information as a guide to therapeutic

interventions. In conclusion, our approach integrates mutation ranking with immune deconvolution

and pathway as well as transcription factor activity analysis, to suggest personalized treatment

strategies. In the future, this will likely be complemented with proteomic information [83] and holds

the promise for enhancing the biological rationale behind individualized therapeutic interventions

even it does not constitute a therapeutic protocol per se. Yet, it serves as a paradigm that supports

and augments the concept of tailored medicine in oncology.

Methods

Patients & clinical specimens

Colorectal cancer patients operated in the surgical department of Gennimatas General Hospital in

Athens, which is part of the Athens Comprehensive Cancer Center (ACCC), participated in the study.

The study was approved by the ethics committee of the Gennimatas Hospital (protocol number

27659). Written informed consent was obtained from all patients. Only patients (n=28) with

histologically confirmed primary colorectal adenocarcinoma and one case of rectal cancer who had

received radiotherapy before surgery, were enrolled in the study cohort. Specimens from each

patient were obtained from both the primary tumor and corresponding normal mucosa, which was

taken from the histologically negative margins (at least 5 cm away from the macroscopically defined

cancerous area) to ensure the absence of cancer cells. Upon surgery, resected samples were placed

in 5-mL RNA*later* (Ambion, Austin, TX, USA) to preserve RNA integrity.

DNA and RNA extraction

DNA and RNA were isolated with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according

to the manufacturer's instructions. Quantification of nucleic acids was performed with a Qubit 2.0

Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) using the Qubit 1X dsDNA BR Assay

(ThermoFisher Scientific) and RNA BR Assay kits (ThermoFisher Scientific) for DNA and RNA,

respectively. In total, 28 paired tumor and normal DNA samples were isolated as well as 28 matching

tumor RNA samples.

Whole Exome Sequencing (WES) and variant calling

DNA libraries were prepared from tumor and matched normal tissues of ACCC patients using Low

Input Exome-Seq Human v6 (Agilent Technologies, Waldbronn, Germany) for exome capture and

analyzed on a NovaSeq 6000 (Paired-End 150bp S2, Illumina, San Diego, CA, USA) in the DKFZ High-

Throughput Sequencing Core Facility. The resulting raw sequencing reads (median: 123 million

reads/sample; spread: 80-148 million reads/sample) were preprocessed based on the internal WES

alignment workflow (AlignmentAndQCWorkflows: 1.2.73-1), as part of the DKFZ-ODCF OTP pipeline

[84]. Briefly, after initial quality control using FastQC, raw reads were aligned to a hg19 customized

version   of   the   1000   genomes   reference   genome

(ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequen

ce/hs37d5.fa.gz) (1KGRef_PhiX), using bwa-mem (version 0.7.15). PhiX-genome sequence was added

to the reference as an additional contig to remove spike-in sequences used to identify potential

cross-contaminations and sample swaps. Marking of duplicated reads was performed with

Sambamba tools (https://github.com/biod/sambamba/releases, version 0.6.5) embedded in the

DKFZ-ODCF Roddy pipeline (https://github.com/DKFZ-ODCF/AlignmentAndQCWorkflows).

High-confidence functional somatic single nucleotide variants (SNVs) and InDels (Insertions &

Deletions) were identified using DKFZ-ODCF SNVCallingWorkflow (https://github.com/DKFZ-

ODCF/SNVCallingWorkflow, version 1.2.166-1) and IndelCallingWorkflow (https://github.com/DKFZ-

ODCF/IndelCallingWorkflow, version 1.2.177-0), respectively, based on the BAM files of each tumor

and its paired control sample. Various quality control criteria were performed to exclude spurious

or/and low quality calls (such as identification of low quality bases, variant allele fraction and

presence of strand bias artifacts), finally assigning a confidence value to each variant. Sample

ACCC_CRC_02 was disregarded in further analysis as COSMIC SBS signature analysis (v3.4-October

2023) flagged this as having a potential PCR/sequencing artifact-based strand bias (prevalence of C>A

alterations indicated presence of COSMIC SBS52; https://cancer.sanger.ac.uk/signatures/sbs/sbs52/,

accessed 2023/12/21). Finally, the ANNOVAR tool [85] (version February 2016) for variant annotation

was utilized for the derivation of all functional mutations defined only by the exonic mutations

including missense, nonsense, splice-site, and stop-loss alterations, by setting a confidence value of

at least 8 out of 10. High-confidence InDels were selected using the Platypus tool [86] implemented in a

custom workflow (https://github.com/DKFZ-ODCF/IndelCallingWorkflow, version 0.8.1.1).

SVRACAS variant annotation and gene prioritization

To efficiently prioritize the resulting high-confidence somatic variants for each patient with the

ultimate goal to identify informative oncogenic alterations, the SVRACAS custom variant scoring

pipeline was applied (https://doi.org/10.5281/zenodo.5636747, accessed 2022/10/24). This

integrative workflow consists of two major steps: variant annotation and prioritization of somatic

functional alterations. Briefly, the SVRACAS ranking module integrates 15 biological resources using

the open-source OpenCRAVAT/OakVar comprehensive workflow for cancer variant annotation and

exploitation [87]. The aggregated output has four evidence categories: "Pathogenicity evidence"

(spanning variant effect prediction tools), a "Cancer evidence" layer (covering cancer variant

interpretation knowledge bases), a "Clinical support" indication (nominating clinically relevant

alterations), and "Expression information" indicating whether an affected gene is expressed (i.e., ≥ 10

read counts in at least 5 samples). An integrated scoring value was then assigned to each variant as a

total SVRACAS score (https://github.com/Jasonmbg/Simple.-Variant-Ranking-Annotation-CAncer-

Score). This holistic prioritization scheme ranges from 0 to 1, representing an average approximation

of the four different types of evidence, based on single nucleotide changes and InDels that occur in

the protein-coding space. Here, a cutoff value ≥ 0.5 was used to include only variants annotated in at

least two of the four evidence categories. Overall, the selection of individual relative cutoffs and

weighted importance of different sources of evidence was based on proposed standard operating

procedures that built on guidelines from cancer consortia such as VICC [88] and ComPerMed [89]. Protein-

coding genes were then mapped to the MANE Select Set (version 0.9) of matched representative

transcripts [90]. Genes that did not map in MANE Select were next assessed for predicted

consequences respective mutations have according to the Sequence Ontology [91]. The aberrant

transcript having the highest predicted impact was selected. The longest transcript was chosen when

the same impact was annotated by Sequence Ontology for several transcripts representing the same

gene. Genes in the ACCC CRC dataset with SVRACAS prioritized mutations were mapped to a curated

list of CRC cancer driver genes in the Integrative OncoGenomics database (IntOGen) [30]

(https://www.intogen.org, v1-2020).

Identification and annotation of Copy Number Alterations

Copy Number Alterations (CNAs) were inferred from the whole exome sequencing data using a

customized workflow (Supplementary File 1). Briefly, CNVkit (version 0.9.3) [92] was utilized with

default parameters to infer discrete copy number segments. Next, tumor cell content and ploidy

were estimated using a method adapted from ACEseq [93]. Absolute copy numbers and allele-specific

copy numbers as well as the decrease in heterozygosity (DH) [94] were estimated segment-wise, for

each possible combination of Tumor cell content and ploidy.

RNA-Sequencing and analysis

RNAseq libraries were prepared using the TruSeq Stranded library protocol (Illumina) and sequencing

was done with a NovaSeq 6000 (Paired-end 100 bp S1) in the DKFZ Sequencing Core Facility to collect

a median of 75 million reads/sample (spread: 65-99 million). Processing of RNA-Seq data was

performed using a customized RNAseq Workflow as a part of the DKFZ – ODCF Roddy pipeline

(https://github.com/DKFZ-ODCF/RNAseqWorkflow, version 1.3.0). After initial QC, the RNAseq raw

reads were aligned to a customized hg19 version of the 1000 genomes reference genome

(1KGRef_PhiX):

(ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequen

ce/hs37d5.fa.gz), by a 2 pass alignment using STAR aligner [95] (version 2.5.3a) and gencode 19 gene

models. PhiX-genome sequence was added to the reference as an additional contig to remove spike-

in sequences that had been included to find potential cross-contaminations and sample swaps.

Additional QC after alignment was performed using samtools *flagstat* command and the RNA-SeQC

tool [96] (version 1.1.8). The featureCounts function [97] as part of the Subread software package [98]

(version 1.5.1) was used to determine the respective raw counts per gene, using gencode 19 gene

models. Downstream RNASeq analysis was performed with R (R version 4.1.0)/Bioconductor

software. The R package EnsDb.Hsapiens.v75 was utilized to annotate gene symbols. Non-specific

intensity filtering was performed to exclude genes with less than 10 read counts in less than five

samples. TMM normalization was conducted using the edgeR R package [99] (version 3.36.0), followed

by incorporating sample weights and increase statistical power using the Voom function. Finally, the

R package immunedeconv [45] (version 2.1.0) was utilized to estimate the composition and density of

immune and stromal infiltration, using the non-log-transformed Transcripts Per Kilobase Million

(TPM) values as input.

TCGA and CPTAC multi-OMICs data

Public data from two CRC cohorts were downloaded from the cBioPortal multi-omics cancer portal

(https://www.cbioportal.org/) i.e., the PanCancer Atlas TCGA Colorectal adenocarcinoma [11] and the

CPTAC Prospective Colon [100, 101] datasets. For both datasets, only samples were processed that had

been profiled for SNVs, CNAs, gene expression (RNAseq). Phosphoproteomic data were available only

from CPTAC. SNVs were retrieved and data analysis was performed using the R package maftools [102]

(version 2.10.0). The significance of differential mutational frequencies between the MSS and MSI

tumors within TCGA and CPTAC cohorts was assessed using Fisher's exact test based on a 2x2

contingency table (adjusted p-value ≤0.1, minimum number of samples harboring mutated genes

≥4), exploiting the mafCompare function of maftools.

Inference of microsatellite instability status in the CRC datasets

We utilized the MSI MANTIS [103] estimators of microsatellite instability provided with the TCGA pan-

cancer dataset, using the following categorization: A TCGA tumor was classified as MSI if the

respective value from MSI MANTIS was higher than 0.6, while tumors with relative values lower than

0.4 were classified as MSS. Any tumors with relative values between 0.4 & 0.6 were denoted as

"Undetermined" and excluded from further analysis. Information about MSI-status of respective

samples was available also for the CPTAC dataset [101] and utilized as provided. Then, the PREMSIm R

package [23] (version 1.0) was employed to infer the microsatellite status based RNA-sequencing data

from all three datasets (TCGA, CPTAC, ACCC), using non-log-transformed Transcripts Per Kilobase

Million values from RNA-sequencing. MSI-L and MSS tumors were grouped together in all datasets

and the term MSS used for this group to distinguish from MSI tumors.

Functional enrichment analysis

Functional Enrichment analysis was performed using the R packages clusterProfiler [104] (version 4.2.2),

and ReactomePA [105] (version 1.38.0) was used for overrepresentation analysis based on REACTOME

molecular pathways. ssGSEA [44] (https://github.com/broadinstitute/ssGSEA2.0) was implemented

based on the ssGSEA projection methodology [106]. The hallmark signatures file (version 7.5.1) was

used in gmt format from the respective Molecular Signatures Database (MSigDB) [107] gene sets

(https://www.gsea-msigdb.org/gsea/msigdb/).   The   script   ssgsea-cli.R

(https://github.com/broadinstitute/ssGSEA2.0) was run in a Windows 10 x64 (build 19044)

environment using command line and the following arguments:

[-i ACCC.CRC.RNASeq.VoomCPM.TMM.Filtered -o ACCC -n none -d ./ssGSEA2.0-

master/db/msigdb/h.all.v7.5.1.symbols.gmt -w 0.75 -c z.score -p 1000 -m 10]. Protein associations

were retrieved and visualized using STRING [36] (https://string-db.org, accessed 2023/12/04), and K-

means clustering was performed to identify functional protein networks.

Pathway, TF and Kinase activity inference analyses

The upper-quartile of processed and log2 transformed RSEM estimated counts from CPTAC RNAseq

data were normalized using the function *normalizeQuantiles* from the limma R package [108] (version

3.50.0). Then, a non-specific intensity filtering procedure was implemented to remove features that

were not expressed at least in the group with the lowest number of samples. The same

preprocessing approach was applied for the TCGA dataset, with two additional steps: as the

estimated raw RSEM counts had been batch effect-corrected, many features assigned by NA values

were removed. Then, the data were log2-transformed if the maximum value was >50. Differential

expression analysis in both datasets (MSI vs MSS) was applied with the limma R package [108].

The PROGENy (Pathway RespOnsive GENes R package [37] (version 1.16.0) was implemented to identify

putative differentially activated molecular pathways, based on the derived differential moderated

statistics extracted from the limma pipeline (i.e., top 100 most responsive genes for each pathway).

PROGENy infers the activity of 14 major signaling pathways, based on the exploitation of consensus

gene signatures and estimated from a large compendium of perturbation experiments [37]. The

DoRothEA-decoupleR pipeline [109] (version 1.6.0) was implemented to elucidate differentially

activated regulatory networks. Relative activities were inferred for every transcription factor (TF)

from the deregulated expression of its respective target genes, using the normalized weighted mean

statistical metric from the decoupleR R package [110] (version 2.1.6). Customized barplots were created

using the R package ggplot2 [111] (version 3.3.5) for the visualization of differentially altered TFs and

pathways.

Phosphoproteomics data from the CPTAC cohort were processed and analyzed to infer differentially

activated signaling cascades between MSS and MSI colon tumors. To this end, normalized

phosphosite ratios were used, representing scaled intensity values. Duplicated entries were removed

and any phosphosites were excluded that had NA values across more than 85% of the total samples.

Differential protein phosphorylation analysis was performed using the limma R pipeline (version

3.50.0). Omnipath-decoupleR [110] and Post-translational Modification Set Enrichment Analysis (PTM-

SEA) [112] with the PTMSigDB signaling pathways' collection (version 2.0.0) were implemented to

determine differentially activated kinases and signaling pathways, respectively. The Omnipath R

package [113] (version 3.2.8) was utilized to retrieve the prior-knowledge interactions composed by

kinase-target relationships, whereas the R package decoupleR [110] (version 2.1.6) was used to

estimate the relative biological activities by application of statistical methods (normalized weighted

mean).

Patient clustering

Unsupervised hierarchical clustering on principal components (HCPC) method was implemented,

based on the R packages FactoMineR [114] (version 2.8) and Factoshiny

(http://factominer.free.fr/graphs/factoshiny.html, version 2.4). The selected scaled pathway and TF

activities per patient were used as input for the *PCA* function. Then, hierarchical clustering on the

first 8 components (accounting ~95% of retained information) was performed using the *HCPC* (kk=Inf,

max=10, nb.clust = -1, consol = TRUE) function. Plots were created using the ComplexHeatmap R

package [115]. Patients were re-ordered within each cluster according to their microsatellite instability

status and selected mutational groups, namely BRAF_mut, KRAS_mut, NRAS_mut, GNAS_mut,

RAS_RAF_GNAS_mut, and RAS_RAF_GNAS_wt.

Identification of actionable alterations for construction of personalized cancer patient maps

Aiming to identify actionable vulnerabilities at the single patient resolution within the ACCC-CRC

cohort, the qualified somatic variants from the SVRACAS scoring scheme were integrated with clinical

implications from the OncoKB precision oncology database [29]. Variant oncogenic effect annotation

was applied to keep only variants characterized as oncogenic or likely oncogenic. Therapeutically

actionable variants in any cancer type were assigned with an OncoKB Therapeutic Level of Evidence

(https://www.oncokb.org/therapeutic-levels, accessed 2022/12/21). Genes carrying actionable

variants were assigned to the treatment baskets of the National Center for Tumor Diseases/German

Cancer Consortium (NCT/DKTK) MASTER (Molecularly Aided Stratification for Tumor Eradication

Research) program, that are based on a set of 472 genes which participate in biological processes

and cellular pathways with established therapeutic recommendations [51].

Reporting summary

Further information on experimental design is available in the Nature Research Reporting Summary

linked to this article.

Data availability

All sequencing data supporting the findings of this study have been uploaded to the European

Genome-Phenome archive (http://www.ebi.ac.uk/ega/), and are available with controlled access of a

DAC via the accession numbers EGAD00001011262 and EGAD00001011263. All computational code,

including R scripts, quarto documents and further information to reproduce the complete

bioinformatics analyses, are available on Zenodo

(https://zenodo.org/doi/10.5281/zenodo.10959699) and Github

(https://github.com/Jasonmbg/ACCC-CRC, https://github.com/Jasonmbg/TCGAPanCan-CRC and

https://github.com/Jasonmbg/CPTAC-Colon) repositories for open-access.

References

1. Kopetz S, *et al.* Encorafenib, Binimetinib, and Cetuximab in BRAF V600E-Mutated Colorectal
Cancer. *N Engl J Med* **381**, 1632-1643 (2019).

633
634 2.   Casak SJ, *et al.* FDA Approval Summary: Tucatinib with Trastuzumab for Advanced
635   Unresectable or Metastatic, Chemotherapy Refractory, HER2-Positive RAS Wild-Type
636   Colorectal Cancer. *Clin Cancer Res* **29**, 4326-4330 (2023).

637
638 3.   Janne PA, *et al.* Adagrasib in Non-Small-Cell Lung Cancer Harboring a KRAS(G12C) Mutation.
639   *N Engl J Med* **387**, 120-131 (2022).

640
641 4.   Siegel RL, Wagle NS, Cercek A, Smith RA, Jemal A. Colorectal cancer statistics, 2023. *CA
642   Cancer J Clin* **73**, 233-254 (2023).

643
644 5.   Sung H, *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality
645   Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**, 209-249 (2021).

646
647 6.   Scharpf RB, *et al.* Genomic Landscapes and Hallmarks of Mutant RAS in Human Cancers.
648   *Cancer Res* **82**, 4058-4078 (2022).

649
650 7.   Dang HX, *et al.* The clonal evolution of metastatic colorectal cancer. *Sci Adv* **6**, eaay9691
651   (2020).

652
653 8.   Househam J, *et al.* Phenotypic plasticity and genetic control in colorectal cancer evolution.
654   *Nature* **611**, 744-753 (2022).

655
656 9.   Loree JM, *et al.* Classifying Colorectal Cancer by Tumor Location Rather than Sidedness
657   Highlights a Continuum in Mutation Profiles and Consensus Molecular Subtypes. *Clin Cancer
658   Res* **24**, 1062-1072 (2018).

659
660 10. Linares J, *et al.* Long-term platinum-based drug accumulation in cancer-associated fibroblasts
661   promotes colorectal cancer progression and resistance to therapy. *Nat Commun* **14**, 746
662   (2023).

663
664 11. Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and
665   rectal cancer. *Nature* **487**, 330-337 (2012).

666
667 12. Mendelaar PAJ, *et al.* Whole genome sequencing of metastatic colorectal cancer reveals prior
668   treatment effects and specific metastasis features. *Nat Commun* **12**, 574 (2021).

669
670 13. de la Chapelle A, Hampel H. Clinical relevance of microsatellite instability in colorectal cancer.
671   *J Clin Oncol* **28**, 3380-3387 (2010).

672
673 14. Hause RJ, Pritchard CC, Shendure J, Salipante SJ. Classification and characterization of
674   microsatellite instability across 18 cancer types. *Nat Med* **22**, 1342-1350 (2016).

675
676 15. Boland CR, *et al.* A National Cancer Institute Workshop on Microsatellite Instability for cancer
677   detection and familial predisposition: development of international criteria for the

678 determination of microsatellite instability in colorectal cancer. *Cancer Res* **58**, 5248-5257
679 (1998).

680
681 16.   Lochhead P, *et al.* Microsatellite instability and BRAF mutation testing in colorectal cancer
682   prognostication. *J Natl Cancer Inst* **105**, 1151-1156 (2013).

683
684 17.   Poynter JN, *et al.* Molecular characterization of MSI-H colorectal cancer by MLHI promoter
685   methylation, immunohistochemistry, and mismatch repair germline mutation screening.
686   *Cancer Epidemiol Biomarkers Prev* **17**, 3208-3215 (2008).

687
688 18.   Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895-
689   2015. *Nat Rev Cancer* **15**, 181-194 (2015).

690
691 19.   Le DT, *et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**,
692   2509-2520 (2015).

693
694 20.   Innocenti F, *et al.* DNA Mutational Profiling in Patients With Colorectal Cancer Treated With
695   Standard of Care Reveals Differences in Outcome and Racial Distribution of Mutations. *J Clin*
696   *Oncol* **42**, JCO2300825 (2023).

697
698 21.   De Smedt L, *et al.* Microsatellite instable vs stable colon carcinomas: analysis of tumour
699   heterogeneity, inflammation and angiogenesis. *Br J Cancer* **113**, 500-509 (2015).

700
701 22.   Cleyle J, *et al.* Immunopeptidomic analyses of colorectal cancers with and without
702   microsatellite instability. *Mol Cell Proteomics* **21**, 100228 (2022).

703
704 23.   Li L, Feng Q, Wang X. PreMSIm: An R package for predicting microsatellite instability from the
705   expression profiling of a gene panel in cancer. *Comput Struct Biotechnol J* **18**, 668-675 (2020).

706
707 24.   Edwards NJ, *et al.* The CPTAC Data Portal: A Resource for Cancer Proteomics Research. *J*
708   *Proteome Res* **14**, 2707-2713 (2015).

709
710 25.   Zampino G, *et al.* Diversity, parental germline origin, and phenotypic spectrum of de novo
711   HRAS missense changes in Costello syndrome. *Hum Mutat* **28**, 265-272 (2007).

712
713 26.   Dankner M, Rose AAN, Rajkumar S, Siegel PM, Watson IR. Classifying BRAF alterations in
714   cancer: new rational therapeutic strategies for actionable mutations. *Oncogene* **37**, 3183-
715   3199 (2018).

716
717 27.   Hu J, *et al.* Allosteric activation of functionally asymmetric RAF kinase dimers. *Cell* **154**, 1036-
718   1046 (2013).

719
28.   Choi J, *et al.* Identification of PLX4032-resistance mechanisms and implications for novel RAF
inhibitors. *Pigment Cell Melanoma Res* **27**, 253-262 (2014).

29.   Chakravarty D, *et al.* OncoKB: A Precision Oncology Knowledge Base. *JCO Precis Oncol* **1**, 1-16
(2017).

30.   Martinez-Jimenez F, *et al.* A compendium of mutational cancer driver genes. *Nat Rev Cancer*
20, 555-572 (2020).

31.   Jan YH, Tan KT, Chen SJ, Yip TTC, Lu CT, Lam AK. Comprehensive assessment of actionable
genomic alterations in primary colorectal carcinoma using targeted next-generation
sequencing. *Br J Cancer* **127**, 1304-1311 (2022).

32.   Alexandrov LB, *et al.* The repertoire of mutational signatures in human cancer. *Nature* **578**,
94-101 (2020).

33.   Dienstmann R, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J. Consensus molecular
subtypes and the evolution of precision medicine in colorectal cancer. *Nat Rev Cancer* **17**, 79-
92 (2017).

34.   Smeets D, *et al.* Copy number load predicts outcome of metastatic colorectal cancer patients
receiving bevacizumab combination therapy. *Nat Commun* **9**, 4112 (2018).

35.   Gillespie M, *et al.* The reactome pathway knowledgebase 2022. *Nucleic Acids Res* **50**, D687-
D692 (2022).

36.   Szklarczyk D, *et al.* The STRING database in 2023: protein-protein association networks and
functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res* **51**,
D638-D646 (2023).

37.   Schubert M, *et al.* Perturbation-response genes reveal signaling footprints in cancer gene
expression. *Nat Commun* **9**, 20 (2018).

38.   Zhang D, *et al.* ASCL2 induces an immune excluded microenvironment by activating cancer-
associated fibroblasts in microsatellite stable colorectal cancer. *Oncogene* **42**, 2841-2853
(2023).

39.   Nguyen TT, *et al.* Mutations in the IFNgamma-JAK-STAT Pathway Causing Resistance to
Immune Checkpoint Inhibitors in Melanoma Increase Sensitivity to Oncolytic Virus
Treatment. *Clin Cancer Res* **27**, 3432-3442 (2021).

40.   Coles GL, *et al.* Unbiased Proteomic Profiling Uncovers a Targetable GNAS/PKA/PP2A Axis in
Small Cell Lung Cancer Stem Cells. *Cancer Cell* **38**, 129-143 e127 (2020).

41.   Ito Y, Bae SC, Chuang LS. The RUNX family: developmental regulators in cancer. *Nat Rev*
Cancer **15**, 81-95 (2015).

42. Mevel R, Draper JE, Lie ALM, Kouskoff V, Lacaud G. RUNX transcription factors: orchestrators
of development. *Development* **146**, (2019).

769
770 43. Xu H, *et al.* Transcription factors in colorectal cancer: molecular mechanism and therapeutic
771   implications. *Oncogene* **40**, 1555-1569 (2021).

772
773 44. Subramanian A, *et al.* Gene set enrichment analysis: a knowledge-based approach for
774   interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550
775   (2005).

776
777 45. Sturm G, *et al.* Comprehensive evaluation of transcriptome-based cell-type quantification
778   methods for immuno-oncology. *Bioinformatics* **35**, i436-i445 (2019).

779
780 46. Guinney J, *et al.* The consensus molecular subtypes of colorectal cancer. *Nat Med* **21**, 1350-
781   1356 (2015).

782
783 47. Nitsche U, *et al.* Mucinous and signet-ring cell colorectal cancers differ from classical
784   adenocarcinomas in tumor biology and prognosis. *Ann Surg* **258**, 775-782; discussion 782-773
785   (2013).

786
787 48. Linnekamp JF, *et al.* Consensus molecular subtypes of colorectal cancer are recapitulated in
788   in vitro and in vivo models. *Cell Death Differ* **25**, 616-633 (2018).

789
790 49. Oshima S, *et al.* Interferon regulatory factor 1 (IRF-1) and IRF-2 distinctively up-regulate gene
791   expression and production of interleukin-7 in human intestinal epithelial cells. *Mol Cell Biol*
792   **24**, 6298-6310 (2004).

793
794 50. Liao W, *et al.* KRAS-IRF2 Axis Drives Immune Suppression and Immune Therapy Resistance in
795   Colorectal Cancer. *Cancer Cell* **35**, 559-572 e557 (2019).

796
797 51. Horak P, *et al.* Comprehensive Genomic and Transcriptomic Analysis for Guiding Therapeutic
798   Decisions in Patients with Rare Cancers. *Cancer Discov* **11**, 2780-2795 (2021).

799
800 52. Prahallad A, *et al.* Unresponsiveness of colon cancer to BRAF(V600E) inhibition through
801   feedback activation of EGFR. *Nature* **483**, 100-103 (2012).

802
803 53. Troiani T, *et al.* Primary and acquired resistance of colorectal cancer cells to anti-EGFR
804   antibodies converge on MEK/ERK pathway activation and can be overcome by combined
805   MEK/EGFR inhibition. *Clin Cancer Res* **20**, 3775-3786 (2014).

806
807 54. Kim D, *et al.* Pan-KRAS inhibitor disables oncogenic signalling and tumour growth. *Nature*
808   **619**, 160-166 (2023).

809
810 55. Pahuja KB, *et al.* Actionable Activating Oncogenic ERBB2/HER2 Transmembrane and
811   Juxtamembrane Domain Mutations. *Cancer Cell* **34**, 792-806 e795 (2018).

812
813 56. Wang C, Jette N, Moussienko D, Bebb DG, Lees-Miller SP. ATM-Deficient Colorectal Cancer
814   Cells Are Sensitive to the PARP Inhibitor Olaparib. *Transl Oncol* **10**, 190-196 (2017).

815
816 57. Behl D, *et al.* Olaparib (O) in patients (pts) with colorectal cancer (CRC) with ATM mutation
817   (mut): Results from the Targeted Agent and Profiling Utilization Registry (TAPUR) study.
818   *Journal of Clinical Oncology* **41**, 122-122 (2023).

819
820 58. Neshat MS, *et al.* Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR.
821   *Proc Natl Acad Sci U S A* **98**, 10314-10319 (2001).

822
823 59. Ma L, *et al.* Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-
824   regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer. *Cancer*
825   *Res* **67**, 7106-7112 (2007).

826
827 60. Fritsche-Guenther R, *et al.* Alterations of mTOR signaling impact metabolic stress resistance
828   in colorectal carcinomas with BRAF and KRAS mutations. *Sci Rep* **8**, 9204 (2018).

829
830 61. Rousseau B, *et al.* The Spectrum of Benefit from Checkpoint Blockade in Hypermutated
831   Tumors. *N Engl J Med* **384**, 1168-1170 (2021).

832
833 62. Westcott PMK, *et al.* Mismatch repair deficiency is not sufficient to elicit tumor
834   immunogenicity. *Nat Genet* **55**, 1686-1695 (2023).

835
836 63. Becker WR, *et al.* Single-cell analyses define a continuum of cell state and composition
837   changes in the malignant transformation of polyps to colorectal cancer. *Nat Genet* **54**, 985-
838   995 (2022).

839
840 64. Li Q, *et al.* RUNX1 promotes tumour metastasis by activating the Wnt/beta-catenin signalling
841   pathway and EMT in colorectal cancer. *J Exp Clin Cancer Res* **38**, 334 (2019).

842
843 65. Pant S, *et al.* Erdafitinib in patients with advanced solid tumours with FGFR alterations
844   (RAGNAR): an international, single-arm, phase 2 study. *Lancet Oncol* **24**, 925-935 (2023).

845
846 66. Jung B, Staudacher JJ, Beauchamp D. Transforming Growth Factor beta Superfamily Signaling
847   in Development of Colorectal Cancer. *Gastroenterology* **152**, 36-52 (2017).

848
849 67. Xue Z, *et al.* MAP3K1 and MAP2K4 mutations are associated with sensitivity to MEK inhibitors
850   in multiple cancer models. *Cell Res* **28**, 719-729 (2018).

851
852 68. Ried T, *et al.* The landscape of genomic copy number alterations in colorectal cancer and
853   their consequences on gene expression levels and disease outcome. *Mol Aspects Med* **69**, 48-
854   61 (2019).

855
856 69. Lord CJ, Ashworth A. BRCAness revisited. *Nat Rev Cancer* **16**, 110-120 (2016).

858   70.   Mestrallet G, Brown M, Bozkus CC, Bhardwaj N. Immune escape and resistance to
859   immunotherapy in mismatch repair deficient tumors. *Front Immunol* **14**, 1210164 (2023).

861   71.   Guo Y, *et al.* Opposing roles of ZEB1 in the cytoplasm and nucleus control cytoskeletal
862   assembly and YAP1 activity. *Cell Rep* **41**, 111452 (2022).

864   72.   Huang Y, Hong W, Wei X. The molecular mechanisms and therapeutic strategies of EMT in
865   tumor progression and metastasis. *J Hematol Oncol* **15**, 129 (2022).

867   73.   Lal N, *et al.* KRAS Mutation and Consensus Molecular Subtypes 2 and 3 Are Independently
868   Associated with Reduced Immune Infiltration and Reactivity in Colorectal Cancer. *Clin Cancer*
869   *Res* **24**, 224-233 (2018).

871   74.   Garralda E, *et al.* MYC targeting by OMO-103 in solid tumors: a phase 1 trial. *Nat Med* **30**,
872   762-771 (2024).

874   75.   Li X, Kaur N, Albahrani M, Karpf AR, Black AR, Black JD. Crosstalk between protein kinase C
875   alpha and transforming growth factor beta signaling mediated by Runx2 in intestinal
876   epithelial cells. *J Biol Chem* **299**, 103017 (2023).

878   76.   Yan X, *et al.* RUNX2 interacts with BRG1 to target CD44 for promoting invasion and migration
879   of colorectal cancer cells. *Cancer Cell Int* **20**, 505 (2020).

881   77.   Tuo Z, *et al.* RUNX1 is a promising prognostic biomarker and related to immune infiltrates of
882   cancer-associated fibroblasts in human cancers. *BMC Cancer* **22**, 523 (2022).

884   78.   Heide T, *et al.* The co-evolution of the genome and epigenome in colorectal cancer. *Nature*
885   **611**, 733-743 (2022).

887   79.   Joanito I, *et al.* Single-cell and bulk transcriptome sequencing identifies two epithelial tumor
888   cell states and refines the consensus molecular classification of colorectal cancer. *Nat Genet*
889   **54**, 963-975 (2022).

891   80.   Khaliq AM, *et al.* Refining colorectal cancer classification and clinical stratification through a
892   single-cell atlas. *Genome Biol* **23**, 113 (2022).

894   81.   Mateo L, *et al.* Personalized cancer therapy prioritization based on driver alteration co-
895   occurrence patterns. *Genome Med* **12**, 78 (2020).

897   82.   Rodon J, *et al.* Genomic and transcriptomic profiling expands precision cancer medicine: the
898   WINTHER trial. *Nat Med* **25**, 751-758 (2019).

900   83.   Wahjudi LW, *et al.* Integrating proteomics into precision oncology. *Int J Cancer* **148**, 1438-
901   1451 (2021).

902
903   84.   Reisinger E, *et al.* OTP: An automatized system for managing and processing NGS data. *J*
904   *Biotechnol* **261**, 53-62 (2017).

905
906   85.   Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-
907   throughput sequencing data. *Nucleic Acids Res* **38**, e164 (2010).

908
909   86.   Rimmer A, *et al.* Integrating mapping-, assembly- and haplotype-based approaches for calling
910   variants in clinical sequencing applications. *Nat Genet* **46**, 912-918 (2014).

911
912   87.   Pagel KA, *et al.* Integrated Informatics Analysis of Cancer-Related Variants. *JCO Clin Cancer*
913   *Inform* **4**, 310-317 (2020).

914
915   88.   Wagner AH, *et al.* A harmonized meta-knowledgebase of clinical interpretations of somatic
916   genomic variants in cancer. *Nat Genet* **52**, 448-457 (2020).

917
918   89.   Froyen G, *et al.* Standardization of Somatic Variant Classifications in Solid and Haematological
919   Tumours by a Two-Level Approach of Biological and Clinical Classes: An Initiative of the
920   Belgian ComPerMed Expert Panel. *Cancers (Basel)* **11**, (2019).

921
922   90.   Morales J, *et al.* A joint NCBI and EMBL-EBI transcript set for clinical genomics and research.
923   *Nature* **604**, 310-315 (2022).

924
925   91.   Cunningham F, Moore B, Ruiz-Schultz N, Ritchie GR, Eilbeck K. Improving the Sequence
926   Ontology terminology for genomic variant annotation. *J Biomed Semantics* **6**, 32 (2015).

927
928   92.   Talevich E, Shain AH, Botton T, Bastian BC. CNVkit: Genome-Wide Copy Number Detection
929   and Visualization from Targeted DNA Sequencing. *PLoS Comput Biol* **12**, e1004873 (2016).

930
931   93.   Kleinheinz K, *et al.* ACEseq – allele specific copy number estimation from whole genome
932   sequencing. *bioRxiv*, 210807 (2017).

933
934   94.   Olshen AB, Bengtsson H, Neuvial P, Spellman PT, Olshen RA, Seshan VE. Parent-specific copy
935   number in paired tumor-normal studies using circular binary segmentation. *Bioinformatics*
936   **27**, 2038-2046 (2011).

937
938   95.   Dobin A, *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21 (2013).

939
940   96.   DeLuca DS, *et al.* RNA-SeQC: RNA-seq metrics for quality control and process optimization.
941   *Bioinformatics* **28**, 1530-1532 (2012).

942
943   97.   Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning
944   sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2014).

946 98.   Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by
947   seed-and-vote. *Nucleic Acids Res* **41**, e108 (2013).

948
949 99.   Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential
950   expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140 (2010).

951
952 100. Zhang B, *et al.* Proteogenomic characterization of human colon and rectal cancer. *Nature*
953   **513**, 382-387 (2014).

954
955 101. Vasaikar S, *et al.* Proteogenomic Analysis of Human Colon Cancer Reveals New Therapeutic
956   Opportunities. *Cell* **177**, 1035-1049 e1019 (2019).

957
958 102. Mayakonda A, Lin DC, Assenov Y, Plass C, Koeffler HP. Maftools: efficient and comprehensive
959   analysis of somatic variants in cancer. *Genome Res* **28**, 1747-1756 (2018).

960
961 103. Kautto EA, *et al.* Performance evaluation for rapid detection of pan-cancer microsatellite
962   instability with MANTIS. *Oncotarget* **8**, 7452-7463 (2017).

963
964 104. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes
965   among gene clusters. *OMICS* **16**, 284-287 (2012).

966
967 105. Yu G, He QY. ReactomePA: an R/Bioconductor package for reactome pathway analysis and
968   visualization. *Mol Biosyst* **12**, 477-479 (2016).

969
970 106. Barbie DA, *et al.* Systematic RNA interference reveals that oncogenic KRAS-driven cancers
971   require TBK1. *Nature* **462**, 108-112 (2009).

972
973 107. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular
974   Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* **1**, 417-425 (2015).

975
976 108. Ritchie ME, *et al.* limma powers differential expression analyses for RNA-sequencing and
977   microarray studies. *Nucleic Acids Res* **43**, e47 (2015).

978
979 109. Holland CH, *et al.* Robustness and applicability of transcription factor and pathway analysis
980   tools on single-cell RNA-seq data. *Genome Biol* **21**, 36 (2020).

981
982 110. Badia IMP, *et al.* decoupleR: ensemble of computational methods to infer biological activities
983   from omics data. *Bioinform Adv* **2**, vbac016 (2022).

984
985 111. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. Springer New York, NY (2009).

986
987 112. Krug K, *et al.* A Curated Resource for Phosphosite-specific Signature Analysis. *Mol Cell*
988   *Proteomics* **18**, 576-593 (2019).

990 113. Turei D, *et al.* Integrated intra- and intercellular signaling knowledge for multicellular omics
991 analysis. *Mol Syst Biol* **17**, e9923 (2021).

992
993 114. Lê S, Josse J, Husson F. FactoMineR: AnRPackage for Multivariate Analysis. *Journal of*
994 *Statistical Software* **25**, 1 - 18 (2008).

995
996 115. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in
997 multidimensional genomic data. *Bioinformatics* **32**, 2847-2849 (2016).

998
999 116. Manders F, *et al.* MutationalPatterns: the one stop shop for the analysis of mutational
1000 processes. *BMC Genomics* **23**, 134 (2022).

1001
1002 117. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat*
1003 *Med* **9**, 811-818 (1990).

1004
1005 118. Boyle EI, *et al.* GO::TermFinder--open source software for accessing Gene Ontology
1006 information and finding significantly enriched Gene Ontology terms associated with a list of
1007 genes. *Bioinformatics* **20**, 3710-3715 (2004).

1008
1009 119. Horak P, *et al.* Assigning evidence to actionability: An introduction to variant interpretation in
1010 precision cancer medicine. *Genes Chromosomes Cancer* **61**, 303-313 (2022).

1011

1012

Acknowledgements

We thank the teams of the DKFZ High-Throughput Sequencing and the OMICS IT Core Facilities for

performing excellent services. We thank Julio Saez-Rodriguez and Aurelien Dugourd (Heidelberg

University), and Marcos Díaz-Gay (University of California San Diego) for support concerning the

implementation of pathway/TF activities, and COSMIC signature analysis, respectively. This study was

supported by the Helmholtz European Partnering Program "Athens Comprehensive Cancer Center

(ACCC)" in the course of a strategic collaboration between the National Hellenic Research Foundation

(NHRF), the G. Gennimatas Hospital, the Medical School of the University of Athens, and the German

Cancer Research Center (DKFZ).

Author contributions

G.N.Z. **clinically assessed or operated patients and coordinated sample acquisition**. S.T., G.N.Z., V.K.,

S.R, Ko.P, and A.P. **collected and processed specimens**. S.T., S.Wo and S.Wi. **oversaw sequencing of**

the samples. E-I.V., K.V., G.W., R.K., Ky.P., and O.P. **carried out the bioinformatics analyses and**

processed data. E-I.V., K.V., J.B., O.P. and S.Wi. **analyzed and interpreted data, and wrote the**

manuscript. E-I.V., A.D-S., G.N.Z., A.P. and S.Wi. **conceived the study**. **All authors participated in**

discussions and interpretation of the data and results.

Funding

Open Access funding enabled and organized by Project DEAL.

Competing interests

The authors declare no competing interests.

Additional Information

Correspondence should be addressed to Efstathios-Iason Vlachavas, Olga Papadodima or Stefan

Wiemann.

1036

Figure Legends

Fig. 1. Mutational landscape in the tumors of the ACCC CRC cohort. Oncoplot presenting scored

genes common with the IntoGen database for 27 ACCC-CRC patients, which were grouped into MSI

and MSS subtypes (MSI_Status). Each column represents a profiled patient and each row a gene of

interest. The alteration frequencies of 54 genes (SNVs, InDels and CNVs) are shown for each patient

and for each gene on the top and on the right barplots, respectively. Genes are sorted based on their

total mutation frequency (depicted on the left). Colored cells indicate respective types of alterations

explained on the right, while gray cells indicate lack of mutations in respective tumors. The

mutational spectrum for each patient showing the relative contribution of each of the six base

substitution types is presented below the top bar plot as a stacked bar chart, using the Mutational

Patterns R package [116] (version 3.4.1).

Fig. 2. **Lollipop plots of REACTOME pathways significantly enriched in MSI vs. MSS tumors of the**

TCGA and CPTAC cohorts. Functional enrichment analysis was performed using clusterProfiler [104]

(v.4.2.2), and ggplot2 (v.3.4.3) was used for the visualization of enrichment results. P-values were

adjusted for multiple comparisons (p.adjust) [117]. Sizes of bullets represent the number of over-

represented genes (Count) in the corresponding pathway and adjusted p-values (p.adjust) are color-

coded. The Rich Factor value (x-axis) describes the relative ratio of the enriched genes in each

pathway, divided by the total number of annotated genes in the respective set (i.e., over-

representation analysis) [118].

Fig. 3. **Significantly differentially activated pathways (A) and transcription factors (TFs) (B) between**

MSI and MSS CRC tumors in three CRC cohorts (CPTAC, TCGA and ACCC). Pathway and TF relative

activities were estimated using PROGENy [37] and DorothEA [109] R packages, respectively. Barplot

visualizes normalized Enrichment Scores (NES) with three different shades of red and blue color

denoting significant pathway/TF activity levels of MSI and MSS tumors in CPTAC, TCGA, and ACCC

CRC datasets, respectively. Horizontal dotted lines at +2/-2 values indicate the lower cut-off values

for statistically significant activities of pathways (A) and TFs (B). Only pathways and TFs found

significantly differentially activated in all three datasets are illustrated. The total numbers of samples

characterizing each CRC group (MSI or MSS) are denoted in parentheses.

Fig. 4. Clustering of ACCC-CRC patients based on integrative transcriptomic analysis. (A) Normalized

enrichment scores (NES) of 7 pathway and 14 TF activities (compare Fig. 3) were used for

unsupervised clustering on principal components (HCPC) with R packages FactoMineR [114] (version

2.8) and Factoshiny (http://factominer.free.fr/graphs/factoshiny.html, version 2.4), distributing ACCC

patients into 3 patient clusters. Features were re-ordered within each Pathway and TF row sub-

clusters using hierarchical clustering ("complete" method, "euclidean" distance), where samples

were firstly grouped based on their cluster ID and subsequently based on their MSI status and

mutational information. Tumors with co-occurring alterations in either in *RAS* and *RAF* or *GNAS* and

RAF genes were assigned to the RAS_RAF_GNAS_mut group. Pathway and TF activities (NES) are

indicated in shades of red (high) vs. blue (low). Asterisks in individual cells denote a significant

activation of the respective pathway/TF in a patient (NES ≥2 or NES ≤-2). (B) Heatmap visualization of

the normalized enrichment scores of hallmarks from MsigDb [107] per patient using ssGSEA [44]. Re-

ordering of rows was performed as described in (A). Hallmark pathway activities as well as immune

or stromal scores (ImmDec: immune deconvolution), representing immune and stromal cell

infiltration in respective tumors, are indicated in shades of red (high) vs. blue (low). Plots were

created using the ComplexHeatmap R package [115].

Fig. 5. SVRACAS prioritized somatic mutations, pathway/TF activities, and immune profiles stratify

ACCC CRC patients to MASTER baskets and personalized therapeutic interventions. (A). Oncoplot

with actionable variants (SVRACAS score ≥0.5) and annotated in the context of sensitivity

(OncoKB_Sens   1-4)   or   resistance   (OncoKB_Res   R1-2)   to   a   targeted   therapy

(https://www.oncokb.org/levels) in any tumor entity. Columns correspond to patients and rows

denote genes and protein-level types of alterations. Types of alterations are indicated in color, while

wild type condition is indicated in gray. Rows were sorted by the percentage of occurring alterations,

whereas columns/samples were firstly grouped based on patient cluster ID, then by microsatellite

instability   status,   and   subsequently   by   selected   driver   genomic   alterations   (i.e.,

KRAS/*BRAF*/RAS_RAF_WT). TMB: Tumor mutational burden. Tumors with co-occurring alterations in

either in *RAS* and *RAF* or *GNAS* and *RAF* genes were assigned to the RAS_RAF_GNAS_mut group. (B).

Mapping of indicated patients to MASTER interventional baskets (MB_assignment in blue) based on

mutated genes that are associated with respective baskets [119] (RME: RAF–MEK–ERK; PAM: PI3K–AKT–

mTOR; DDR: DNA damage repair; TK: tyrosine kinases; OTH: other; DEV: developmental regulation;

CC: cell cycle). Mapping to the immune evasion (IE) basket was based on the presence of MSI and/or

high TMB. MB_Assignment in white: not assigned. (C). Molecular rationale for suggested

personalized therapeutic interventions for two ACCC_CRC patients. The first series of boxes

(aberrations) illustrates actionable variants (in orange) and Tumor Mutational Burden (TMB) as well

as MSI-related COSMIC SBS signatures [32]. The second series (activities) presents perturbed molecular

pathways, TF-MYC, and stromal and immune signatures, with boxes framed in shades of red or blue

to indicate either high or low activity levels and high or low scores, respectively. The final series

(Baskets & drugs) shows interventional baskets [119], with suggested drugs, based on a comprehensive

analysis of actionable genomic variants, molecular pathway/TF activities, and immune profiles.

Supplementary Table Legends

1104

Supplementary Table 1: Patient characteristics of the ACCC CRC patient cohort. Age (patient age at

the time of diagnosis), sex (F: female; M: male), tumor_location, histological_subtype

(adenoCa_int_diff: adeno carcinoma with intermediary differentiation; adenoCa_NOS: adeno

carcinoma not otherwise specified; LVI: lymphovascular invasion; src-mucinous_adenoCa: serrated

mucinous adenocarcinoma; adenoCa_low_diff: adeno carcinoma with low differentiation;

desmin_adenoCa: desmin-positive adeno carcinoma), T (tumor), N (lymph node), and M (distant

metastasis) status, and mutation status in *RAS, RAF, GNAS* genes is provided (NA: not analyzed).

Supplementary Table 2. MSI-status (MSI MANTIS [103]) provided with the TCGA dataset and PreMSIm

[23] predictions for 434 patients of the TCGA PanCan COAD/READ cohort [11]. [1] MSI-high

(Microsatellite instability); [0] MSI-low/MSS (Microsatellite stability).

Supplementary Table 3. MSI-status provided with the CPTAC dataset and PreMSIm [23] predictions

for 79 patients of the CPTAC CRC cohort [24]. [1] MSI-high (Microsatellite instability); [0] MSI-low/MSS

(Microsatellite stability).

Supplementary Table 4. Tumor mutational burden, total numbers of somatic mutations, numbers

of SVRACAS scored mutations, MSI-status prediction and probability for 28 patients of the ACCC

CRC cohort. Microsatellite status was estimated using PreMSIm [23]. [1] MSI-high (Microsatellite

instability); [0] MSI-low/MSS (Microsatellite stability).

Supplementary Table 5. List of somatic mutations in the ACCC patients, with variant annotation

information from OpenCRAVAT/OakVar, along with the SVRACAS scoring estimates. Ref_Base:

Base in reference sequence; Alt_Base: Base in tumor; Selected_Transcript: Genes with alterations

were initially mapped to MANE Select [90] (version 0.9). Genes that did not map in MANE Select were

next assessed for predicted consequences respective mutations have according to the Sequence

Ontology [91]. The aberrant transcript having the highest predicted impact was selected. The longest

transcript was chosen when the same impact was predicted by Sequence Ontology for several

transcripts representing the same gene; cChange: position and consequence of alteration compared

to reference nucleodide sequence; pChange: position and consequence of alteration compared to

reference protein sequence; SVRACAS_Score: Pathogenicity and score assigned to every alteration

using the SVRACAS scoring pipeline (https://github.com/Jasonmbg/Simple.-Variant-Ranking-

Annotation-CAncer-Score).

Supplementary Table 6. List of somatic mutations in TCGA patients, with variant annotation

information from OpenCRAVAT/OakVar, along with the SVRACAS scoring estimates. Ref_Base:

Base in reference sequence; Alt_Base: Base in tumor; Selected_Transcript: Genes with alterations

were initially mapped to MANE Select [90] (version 0.9). Genes that did not map in MANE Select were

next assessed for predicted consequences respective mutations have according to the Sequence

Ontology [91]. The aberrant transcript having the highest predicted impact was selected. The longest

transcript was chosen when the same impact was predicted by Sequence Ontology for several

transcripts representing the same gene; cChange: position and consequence of alteration compared

to reference nucleodide sequence; pChange: position and consequence of alteration compared to

reference protein sequence; SVRACAS_Score: Pathogenicity and score assigned to every alteration

using the SVRACAS scoring pipeline (https://github.com/Jasonmbg/Simple.-Variant-Ranking-

Annotation-CAncer-Score).

Supplementary Table 7. List of somatic mutations in CPTAC patients, with variant annotation

information from OpenCRAVAT/OakVar, along with the SVRACAS scoring estimates. Ref_Base:

Base in reference sequence; Alt_Base: Base in tumor; Selected_Transcript: Genes with alterations

were initially mapped to MANE Select [90] (version 0.9). Genes that did not map in MANE Select were

next assessed for predicted consequences respective mutations have according to the Sequence

Ontology [91]. The aberrant transcript having the highest predicted impact was selected. The longest

transcript was chosen when the same impact was predicted by Sequence Ontology for several

transcripts representing the same gene; cChange: position and consequence of alteration compared

to reference nucleodide sequence; pChange: position and consequence of alteration compared to

reference protein sequence; SVRACAS_Score: Pathogenicity and score assigned to every alteration

using the SVRACAS scoring pipeline (https://github.com/Jasonmbg/Simple.-Variant-Ranking-

[Annotation-CAncer-Score).](https://github.com/Jasonmbg/Simple.-Variant-Ranking-Annotation-CAncer-Score)

Supplementary Figure Legends

Supplementary Fig. 1. Relative contribution of COSMIC mutational signatures in each patient of the

ACCC-CRC cohort. Single base substitution (SBS) signatures [32] were assessed using the

SigProfilerAssignment web tool and the ggplot2 R package (v.3.4.3). Signatures accounting for similar

biological processes were grouped together into the same "color" representation in the barplot,

based on the following scheme: MSI-related signatures [SBS6, SBS15, SBS20, SBS26] and POLE-

related mutational procedures [SBS10a and SBS10b].

Supplementary Fig. 2. K-means clustering of STRING protein-protein interaction network of high

mutational frequency genes in MSI versus MSS colorectal cancer. The network was generated using

the STRING database (version 12.0) with an input of 60 genes identified to have a higher mutational

frequency in MSI compared to MSS colorectal cancer (CRC) tumors. Interactions were considered at a

confidence threshold score ≥0.7. The network was clustered into groups using k-means clustering.

Respective genes within clusters are color coded. Solid lines indicate direct functional or/and physical

protein associations within clusters, while dotted lines represent interactions between clusters. Gene

symbols are indicated in the network.

Supplementary Fig. 3. Kinase and Pathway Activities, and GNAS Alterations in MSS and MSI CRC

Tumors from the CPTAC cohort. (A) Barplot of the top-10 most activated (red bars) and most

repressed (blue bars) kinases between MSS and MSI colorectal cancer (CRC) tumors, based on

differential phospho-proteomic analysis from the CPTAC patient cohort, with normalized enrichment

scores (NES) from the OmnipathR-decoupleR pipeline [110]. Inferred kinase activities are indicated in

red (higher in MSS, NES ≥2) and blue (higher in MSI, NES ≤-2) colors, respectively. (B) Barplot of

estimated pathway activities, showing top-selected activated (red) and repressed (blue) pathways,

with NES derived from PTM-SEA [112] using PTMSigDB pathway collection (version 2.0.0). (C) Heatmap

of GNAS copy number alterations (from GISTIC 2.0) alongside scaled gene expression values (RNAseq

data) of CPTAC-CRC tumors, stratified by their MSI status. (D) Violin plot detailing GNAS normalized

expression values between MSS and MSI tumors of CPTAC cohort.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5