

Next generation sequencing identifies ‘interactome’ signatures in relapsed and refractory metastatic colorectal cancer

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Background: In the management of metastatic colorectal cancer (mCRC), *KRAS*, *NRAS* and *BRAF* mutational status individualizes therapeutic options and identify a cohort of patients (pts) with an aggressive clinical course. We hypothesized that relapsed and refractory mCRC pts develop unique mutational signatures that may guide therapy, predict for a response and highlight key signaling pathways important for clinical decision making.

Methods: Relapsed and refractory mCRC pts (N=32) were molecularly profiled utilizing commercially available next generation sequencing (NGS) platforms. Web-based bioinformatics tools (Reactome/Enrichr) were utilized to elucidate mutational profile linked pathways-networks that have the potential to guide therapy.

Results: Pts had progressed on fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab, cetuximab and/or panitumumab. Most common histology was adenocarcinoma (colon N=29; rectal N=3). Of the mutations TP53 was the most common, followed by *APC*, *KRAS*, *PIK3CA*, *BRAF*, *SMAD4*, *SPTA1*, *FAT1*, *PDGFRA*, *ATM*, *ROS1*, *ALK*, *CDKN2A*, *FBXW7*, *TGFBR2*, *NOTCH1* and *HER3*. Pts had on average had ≥ 5 unique mutations. The most frequent activated signaling pathways were: HER2, fibroblast growth factor receptor (FGFR), p38 through BRAF-MEK cascade via RIT and RIN, ARMS-mediated activation of MAPK cascade, and VEGFR2.

Conclusions: Dominant driver oncogene mutations do not always equate to oncogenic dependence, hence understanding pathogenic ‘interactome(s)’ in individual pts is key to both clinically relevant targets and in choosing the next best therapy. Mutational signatures derived from corresponding ‘pathway-networks’ represent a meaningful tool to (I) evaluate functional investigation in the laboratory; (II) predict response to drug therapy; and (III) guide rational drug combinations in relapsed and refractory mCRC pts.

Keywords: Metastatic colorectal cancer (mCRC); *RAS*; *BRAF*; next generation sequencing (NGS); mutational signatures; interactome

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Introduction

Colorectal cancer (CRC) remains a significant healthcare issue representing the 4th most frequently diagnosed cancer and the second leading cause of cancer mortality in the

United States. In 2015 alone, nearly 133,000 estimated new cases of CRC were diagnosed, reflecting eight percent of all new cancer cases. An estimated 49,700 cancer deaths from CRC is predicted in 2015 (1). While the burden of disease is undeniably evident, therapeutic progress is

being made in the treatment of CRC. The Center for Disease Control (CDC) has reported that between 2008 and 2011, the incidence of CRC has decreased at a rate of 4% per year. Morality rate has also decreased by nearly 35% in 1990–2007 and in 2011, down by 47% (1,2). This improvement in incidence and overall mortality rate is a function of at least three known improvements in the approach and management of CRC. First, a concerted effort for improved cancer prevention across the nation. Second, increased community awareness through active colorectal screening protocols resulting in prompt diagnosis and referral. Third, a recent surge of FDA approved novel chemotherapeutic and biologic treatment modalities have increased the oncologists' therapeutic repertoire to manage this disease. Metastatic colorectal cancer (mCRC) represents 20% of newly diagnosed CRC and through a multidisciplinary approach with the utilization of systemic backbone chemotherapy, biologic, targeted therapy and evolving surgical and radiologic interventions, the overall survival (OS) has improved to greater than thirty months.

In regards to the management of mCRC, expanded RAS mutation testing to include KRAS and NRAS codons 12 and 13 (exon 2), 59 and 61 (exon 3), and 117 and 146 (exon 4) is a predictive biomarker and guides therapeutic options (3,4). *BRAF V600E* mutation has also been validated to be a prognostic biomarker identifying a unique cohort of patients (pts) who will have a more aggressive clinical course (5). Recently, an exciting relationship between microsatellite instability high (MSI-H) and an impressive response to immune checkpoint blockade with anti-PD1 therapy in mCRC pts has been established (6). This reflects the hypothesis that MSI-H CRC harbors thousands of somatic mutations generating neo-antigens that are likely susceptible to immune checkpoint blockade via anti-PD1 therapy. These data reaffirm that genomics continues to emerge as a critical component in the management of advanced CRC pts. The seminal work conducted by the Human Genome Project has paved the way for subsequent Cancer Genome Atlas investigations that surveyed the specific genetic make-up of numerous malignancies in order to promote a deeper molecular understanding of cancer and provided potential clinical insights (7).

Next generation sequencing (NGS) has now become readily available and markedly affordable facilitating widespread clinical accessibility of 'personalized' genetic aberrations in advanced cancer pts. Although we can all agree NGS offers a wealth of genomic information, its implications in ensuring true precision has not been

completely defined. Therefore, 'precision medicine' in oncology today should be defined as an evolving analysis with goals of correlating patient specific genetic data with prognosis, predicting response and resistance to therapy to guide treatment decisions for an individual patient. Considering that tumors consist of distinct molecular dynamics, comprised of DNA mutations, chromosomal abnormalities, gene and epigenetic expressions and proteomics reflects vast tumor heterogeneity. The new taxonomy for cancer is clearly here to stay where histology alone is no longer sufficient. Therefore, the incorporation of large scale genomic sequencing is appropriately redefining how we categorize cancer today and for the medical oncologist there is a need to accurately elucidate what role it will play in decision making for precision medicine.

A large global consortium has characterized CRC into four distinct 'consensus molecular subtypes' reflecting a new era of oncologic taxonomy for CRC (8). This paradigm shifting approach to CRC will undoubtedly translate into innovative 'personalized' clinical trial design that investigates potentially actionable genomic variants while simultaneously seeking biomarker validation in carefully pre-selected cohorts. We believe that as medical oncologists investigating specific mutational profiles can be correlated with clinical phenotypes however consideration of the entire 'molecular signature' to promote translational and functional studies is a high priority. These studies can serve as a means to better understand the biology of CRC, identify novel targets, unveil anticipated responses and acquired mechanisms of resistance to targeted therapy. Risk-based stratification of pts promotes innovative genomic driven prospective clinical trials. The landscape of clinical trials has already dramatically shifted to a genomic focus for both ASCO and NCI (9,10). We sought to further investigate the molecular signatures of our cohort of relapsed and refractory mCRC pts through the use of NGS platforms to evaluate pathway-network analysis to highlight how NGS data can be incorporated into the clinic today. We hypothesized that relapsed and refractory mCRC pts possess unique mutations that drive distinct aberrant pathways-networks that help understand pathobiology and generate novel therapies to be evaluated in early phase clinical trials.

Methods

Thirty-two relapsed and refractory mCRC pts (N=32) were profiled by commercially available NGS platforms: Caris Molecular Intelligence (IHC, FISH/CISH, NGS)

and/or Foundation One (NGS, copy number). Tumor samples were formalin-fixed, paraffin-embedded (FFPE) blocks from diagnostic biopsies and/or metastatic sites. Each mutational profile reported histology, primary and/or metastatic site, biopsy location, gene mutation (G-site/G-mutation; P-site/P-mutation), domain, topology, and mutation count/gene. All patient information was de-identified. We then utilized web-based bioinformatics tools (Enrichr/Reactome) to carefully analyze each mutational profile identifying common and/or novel signaling as well as potential feedback loop pathway-networks, representing individual 'mutational signatures' (11-13).

Enrichr is a free web-based gene signature search engine that contains over 90,000 annotated signatures allowing for downstream signal and functional analytics. It encompasses over 30 gene-set libraries and various interactive visualization approaches to informatively display enrichment results via JavaScript library Data Driven Documents (D3) (12,13). This tool provides a visualization summary of known pathways based on a collective gene function list. Reactome is a free, open-source, curated and peer reviewed pathway database available for online use. This bioinformatics database provides a mechanism for biologic interpretation and visualization models for network pathway analysis. Pathway network interactions and annotations are authored by expert biologists in the field as well as cross-referenced to numerous bioinformatics databases, such as NCBI Gene, Ensembl and UniProt databases, the UCSC and HapMap Genome Browsers, the KEGG Compound and ChEBI small molecule databases, PubMed, and Gene Ontology (11,13). Through the use of Reactome and Enrichr, we were able to systematically visualize anticipated pathway networks based upon patient specific molecular profiles. These bioinformatics tools allow for prompt visualization and analysis of involved signaling pathway networks fostering further hypothesis generation for biomarker development.

Results

Mutational frequencies in relapsed and refractory mCRC

All mCRC pts included in this analysis had progressed on fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab, cetuximab or panitumumab. Adenocarcinoma was the most common histology followed by squamous cell carcinoma (colon N=29; rectal N=3). In our cohort of thirty-two relapsed and refractory mCRC pts, we identified the

following oncogenes and genetic aberrations in highest frequency—*TP53*, *APC*, *KRAS*, *PIK3CA*, *BRAF*, *SMAD4*, *SPTA1*, *FAT1*, *PDGFRA*, *ATM*, *ROS1*, *ALK*, *CDKN2A*, *FBXW7*, *TGFBR2*, *NOTCH1* and *HER3* (Figure 1, Table 1). Most patients had on average >5 unique gene mutations. Five patients were identified with MSI-H. High mutational burden was not predictive for PD-1 (n=5) or PD-L1 (n=1) positivity (Figure 1).

The 'interactome' in mCRC

There are no simple genotype-phenotype relationships in mCRC as highlighted by the *BRAF* mutation status. In refractory melanoma vemurafenib showed a 78% response rate but in mCRC the response rate was 5% (14,15). Thus, a dominant oncogene mutation does not imply oncogenic dependence, as pathway-network status is distinct to each tumor type. Hence, biologic context referred to here as the 'interactome' i.e., network of activated pathways and feedback loops, confound isolated targeting of a specific genetic aberration. Enrichr and Reactome tools were utilized to interrogate signaling pathways concomitantly represented in our cohort of mCRC pts based upon their specific mutational profiles (Figure 2). Collectively the interactome generated and identified a map of signaling nodes that predict for activation of four interacting pathways in mCRC: (I) Erb-B2 receptor kinase 2 (HER2); (II) fibroblast growth factor receptor 1 (FGFR1); (III) p38 activation through BRAF-MEK cascade via Ras-like protein in tissues (RIT) and Ras-like protein in neurons (RIN) GTPases; (IV) ARMS-mediated activation of MAPK cascade; and (V) VEGFR2 (Figure 2). In addition, there were eight cases with DNA-repair defects with mutations in *ATM*, *CHEK2*, *FANCA* and/or *BRCA2* that may warrant further investigation with PARP inhibition (Table 1) (16,17). Whether the development of somatic mutations in DNA repair genes are secondary to heavy pretreatment with chemotherapy would need to be investigated, thereby identifying an expected tumor evolution signature that could potentially be exploited with PARP inhibition in refractory mCRC. Tumor evolution was further appreciated within our cohort of patients when NGS data from new liver metastasis was obtained in a patient with expanded *RAS* wild-type mCRC heavily pretreated with chemotherapy, anti-VEGF therapy and anti-EGFR therapy that subsequently developed coexistent *KRAS* (Q61L) plus *NRAS* (Q61H) mutations (Table 1). To highlight both the importance and complexity of the "interactome" as a critical

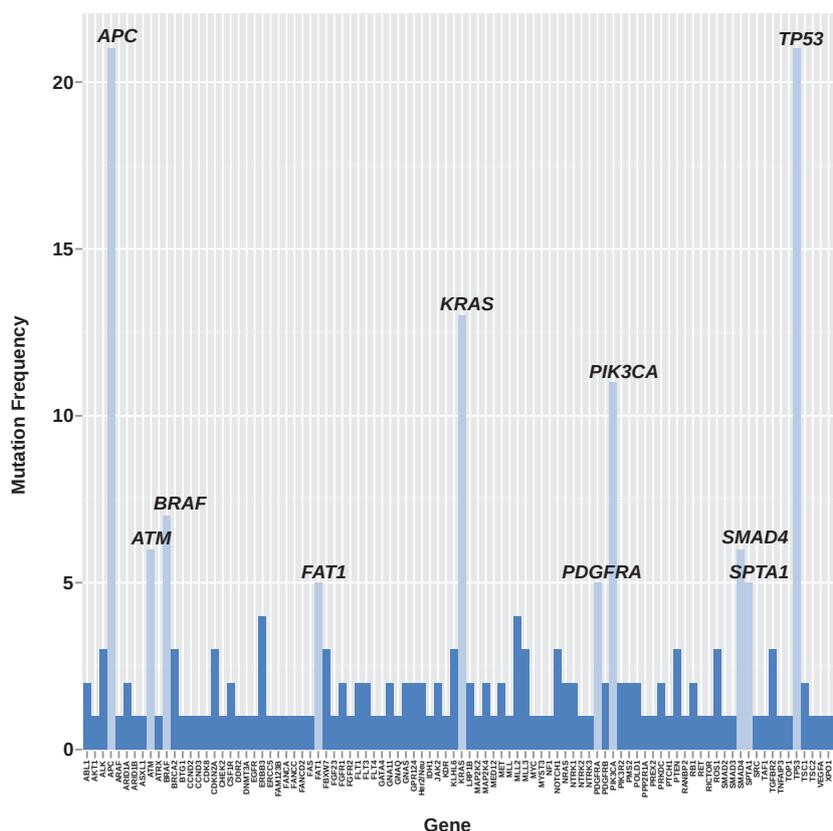


Table 1 Novel mutational signatures and corresponding activated pathway(s) in relapsed/refractory mCRC

Relapsed/refractory mCRC (N=32)	Mutational signature via NGS	Activated pathway(s) via Enrichr analysis
Colon 1	<i>ABL1, APC, ATM, CHEK2, ERBB3, GNA11, GNAQ, Her2/neu, JAK2, NF1, PIK3CA</i>	Activation of ERBB2 signaling; activation of FGFR
Colon 2	<i>APC, BRCA2, FAT1, FLT1, KRAS, MYC, NTRK1, PMS2, TP53, ZNF703</i>	Signaling to p38 via RIT and RIN; ARMS mediated activation
Colon 3	<i>BRCA2, CDK8, DDR2, EGFR, FLT1, FLT3, GPR124, MLL, MLL3, NRAS, PRKDC, SMAD4, SPTA1, SPTA1, TP53</i>	Activation of ERBB2 signaling
Colon 4	<i>APC, FLT3, KRAS, TP53</i>	RAF/MAP kinase cascade
Colon 5	<i>CCND2, GNAS, GNAS, PDGFRA, PREX2, RICTOR, SPTA1, TP53</i>	Activation of ERBB2, activation of FGFR; PI3K through ERBB2
Colon 6	<i>TP53</i>	P53 dependent G1 DNA damage response; G1/S damage checkpoints
Colon 7	<i>APC, PIK3CA, PTEN</i>	PI3K/Akt activation; PI3K events in ERBB2 signaling; downstream TCR signaling
Colon 8	<i>BRAF, TP53</i>	Signaling to p38 via RIT and RIN; ARMS mediated activation
Colon 9	<i>APC, CSF1R, KRAS, NOTCH1, PDGFRA, TP53</i>	Signaling by ERBB2 activation
Colon 10	<i>ALK, APC, ATM, BTG1, FBXW7, KLHL6, LRP1B, MED12, MLL2, PDGFRB, PPP2R1A, SMAD4, SPTA1, TP53, TSC1</i>	G1/S DNA damage checkpoints
Colon 11	<i>APC, CSF1R, KRAS, MET, PDGFRB, PIK3CA, PTCH1</i>	Signaling by ERBB2; signaling by FGFR
Colon 12	<i>BRAF, KDR, TP53</i>	VEGFR2 mediated cell proliferation; ARMS mediated activation; signaling to p38 via RIT and RIN
Colon 13	<i>APC, KRAS, TP53</i>	RAF/MAP kinase cascade
Colon 14	<i>APC, ARAF, ARID1A, FAS, FAT1, MAP2K4, MLL2, MLL2, PDGFRA, PIK3CA, PIK3R2, PTEN, RB1, TGFB2, TOP1</i>	FCERI signaling, PI3K pathway
Colon 15	<i>APC, ARID1A, DNMT3A, FAT1, MAP2K4, MLL2, MLL2, PDGFRA, PIK3R2, RB1, TGFB2</i>	FCERI signaling
Colon 16	<i>KRAS, SMAD4, TP53</i>	RAF/MAP kinase cascade
Colon 17	<i>ERCC5, FANCA, KRAS, LRP1B, LRP1B, MET, PMS2, SMAD4, TP53</i>	DNA repair; RAF/MAP kinase cascade; signaling to p38 via RIT and RIN
Colon 18	<i>ALK, APC, GNAS, Her2/Neu, ROS1, TP53</i>	ERBB2 signaling
Colon 19	<i>APC, BRAF</i>	P38 via RIT and RIN
Colon 20	<i>APC, FAM123B, KLHL6, KRAS, NOTCH1, NTRK2, PIK3CA, POLD1, SMAD4, TAF1, TGFB2, TP53, XPO1</i>	Loss of function of TGFB2; SMAD signaling
Colon 21	<i>APC, CCND3, FANCC, FANCD2, KRAS, MAP2K2, PIK3CA, POLD1, ROS1, SPTA1, TP53, VEGFA</i>	VEGFR2 mediated cell proliferation; VEGFA-VEGFR2 pathway
Colon 22	<i>AKT1, FBXW7, KRAS, NTRK3, TP53</i>	ERBB2 activation

Table 1 (continued)

Table 1 (continued)

Relapsed/refractory mCRC (N=32)	Mutational signature via NGS	Activated pathway(s) via Enrichr analysis
Colon 23	<i>ABL1, APC, APC, CDKN2A, ERBB3, KRAS, NOTCH1, RET, SRC, TP53</i>	ERBB2 activation, VEGFR2 mediated cellular proliferation
Colon 24	<i>APC, ATM, BRAF, CDKN2A, NTRK1, TP53</i>	P38 signaling via RIT and RIN; ARMS mediated activation
Colon 25	<i>APC, ATM, BRCA2, FGFR1, PIK3CA, TP53</i>	FGFR activation
Colon 26	<i>ALK, APC, FGFR1, KRAS, PIK3CA, TP53</i>	FGFR activation; IGF1R activation
Colon 27	<i>APC, ARID1B, ASXL1, BRAF, ERBB3, FAT1, FBXW7, FLT4, GATA4, MLL3, PIK3CA, SMAD2, SMAD3, SPTA1, TNFAIP3, TSC1, TSC2</i>	FGFR activation
Colon 28	<i>APC, ATM, BRAF, CDKN2A, ERBB3, FGFR2, GNA11, JAK2, ROS1, TP53</i>	FGFR activation
Colon 29	<i>BRAF</i>	P38 signaling via RIT and RIN; ARMS mediated activation
Rectal 1 (adeno)	<i>PIK3CA</i>	FGFR1 activation; CD28 co-stimulation
Rectal 2 (adeno)	<i>APC, ATM, ATRX, FAT1, FGF23, GPR124, IDH1, KLHL6, KRAS, MLL2, MLL3, MYST3, NRAS, PDGFRA, PRKDC, PTEN, RANBP2, SMAD4, ZNF703</i>	ERBB2 activation, FGFR activation
Rectal 3 (squam)	<i>PIK3CA</i>	FGFR1 activation; CD28 co-stimulation

mCRC, metastatic colorectal cancer; NGS, next generation sequencing; FGFR, fibroblast growth factor receptor; RIT, Ras-like protein in tissues; RIN, Ras-like protein in neurons; FCER1, Fc epsilon receptor.

patient with exon 2/T68M (VUS). HER3 is a kinase depleted co-receptor that is successful in downstream signaling after forming heterodimers with other ErbB family members in order to bind its ligand heregulin (19) which activates the PI3K pathway (20). Preclinical work has revealed that HER3 overexpression by IHC is a prognostic marker for inferior survival in CRC. A phase I study in refractory cancer pts investigating MEHD7945A, a human IgG1 antibody with dual binding to HER3 and EGFR, found 4 of 12 CRC pts developed stable disease for >8 weeks. Currently a phase II study is actively recruiting pts with refractory *KRAS* wild type mCRC to treatment arms with FOLFIRI plus MEHD7945A versus FOLFIRI plus cetuximab (NCT01652482). Dual targeting of HER2/HER3 plus PI3K/AKT/mTOR pathway is an approach for these pts. Previous work established *PIK3CA* mutations as a prognostic biomarker in CRC considering that aspirin use in *PIK3CA* mutated CRC results in longer survival compared to wild-type *PIK3CA* CRC, supporting secondary prevention with aspirin therapy (21). Due to its effect via PI3K/AKT/mTOR pathway on cell growth and survival and

representing a compensatory feedback resistance pathway in response to anti-EGFR therapy, this represents a key target in mCRC. Early phase clinical trials combining dual PI3K/mTOR inhibition and/or cytotoxic chemotherapy with anti-EGFR therapy are underway to ascertain the potential outcome of targeting this alteration in mCRC (22,23).

Implications for FGFR Pathway in mCRC

FGFR1, *FGFR2*, and *FGF23* aberrations were identified by NGS analysis in wild type and mutated *KRAS* pts with mCRC. Brivanib alaninate, the L-alanine ester prodrug of brivanib, an oral, potent and small molecule inhibitor of VEGFR/FGFR tyrosine kinases (24) was tested in a phase III trial of 750 pts randomized to cetuximab plus brivanib alaninate versus cetuximab plus placebo in pts with metastatic chemotherapy refractory wild type *KRAS* mCRC (CO.20) (25). Pts enrolled on study received at least one prior line of therapy with no prior exposure to anti-EGFR therapy. The cetuximab plus brivanib had a statistically significant progression free survival (PFS) (5.0 vs. 3.4 months;

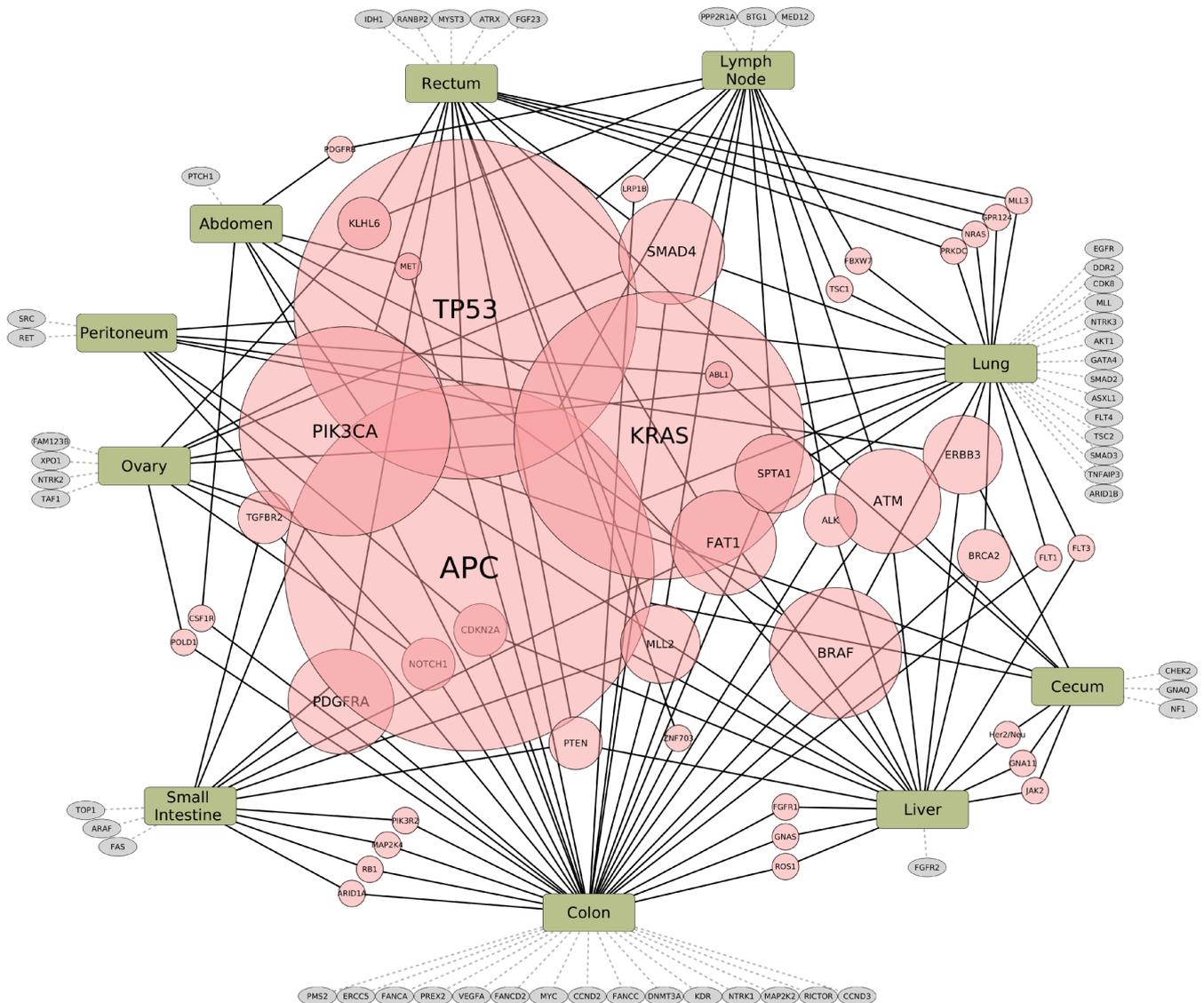


Figure 2 Map of mutation frequencies and the ‘interactome’ in relapsed and refractory mCRC patients. mCRC, metastatic colorectal cancer.

HR, 0.72; $P < 0.001$) and a partial response rate of 13.6% *vs.* 7.2% favoring the combination arm ($P = 0.004$). However, there was no statistically significant improvement in OS (8.8 *vs.* 8.1 months; $P = 0.12$) with worsening grade 3 non-hematologic toxicities with cetuximab plus brivanib. Although a ‘negative’ study, there were interesting findings. For example, the discordance between PFS and OS and the poor quality of life metrics likely has less to do with FGFR targeted therapy but more reflective of an unselected patient population entering a targeted clinical trial. To date, there are no validated predictive biomarkers for the

appropriate selection of patients who will respond to *FGFR* inhibition in mCRC. Our data reveal a cohort of mCRC pts with *FGFR1*, *FGFR2* and *FGF23* mutations with associated *FGFR* pathway activation. Considering the PFS results in CO.20, there is indeed a cohort of pts within the total cohort that had clear response to therapy with *FGFR* inhibition, however these pts were not preselected based on a validated biomarker. Retrospective analysis of the CO.20 trial may serve to highlight and validate a biomarker of response in mCRC pts with the utilization of IHC, FISH and NGS to correlate with response. Further investigation

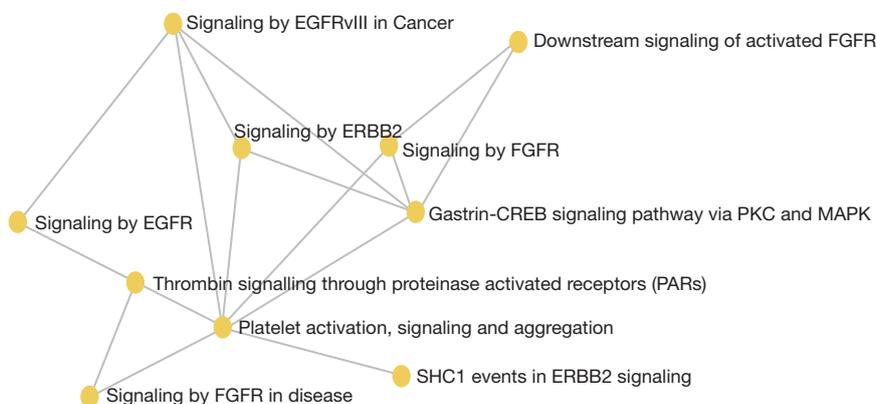
EB is a 51 year-old Caucasian male with all RAS WT, MSI-H (MSH6 mutated, IHC negative) mCRC who failed multiple lines of chemotherapy and biologic therapy including FOLFOX6/Avastin, FOLFIRI + cetuximab, FOLFOX4 + panitumumab, irinotecan + ziv-aflibercept, irinotecan + ramucirumab and regorafenib. He was referred to the New Therapeutics Program at WCC due to progressive hepatic metastasis. NGS was sent on fresh tumor tissue revealing:

Mutation profile Post
Chemotherapy and
Biologic Therapy



ABL1
APC
ATM
CHEK2
ERBB3
GNA11
GNAQ
HER2/neu
JAK2
NF1
PIK3CA
PD-1+

Reactome identifies activated pathway networks based on new mutational signature:



Clinical Implications

1. NGS profiling identifies a mutational signature resistant to anti-EGFR targeted therapy regardless of all RAS WT status.
2. MSI-H status suggests benefit of anti-PD-1 therapy on clinical trial.
3. HER2/neu mutation identifies a candidate for dual HER2 targeted clinical trial.
4. Reactome highlights activated ERBB2 pathway supporting use of targeted therapy against HER2/neu.
5. CHEK2 mutation suggests potential benefit from PARP inhibition.

Figure 3 Case study: all RAS WT, MSI-H mCRC patient progressed on chemotherapy and biologic therapy. MSI-H, microsatellite instability high; mCRC, metastatic colorectal cancer.

using NGS and concomitant pathway analysis to stratify mCRC pts who would benefit most from FGFR inhibition is warranted.

Anti-angiogenic signature in mCRC

In our cohort of relapsed and refractory *RAS* wild type and mutated mCRC pts, we identified *PDGFRA* as one of the most common mutation frequencies (Figure 1) and in conjunction with pathway analysis we noted 3 pts with *PDGFRA* mutations and corresponding FGFR pathway activation (Table 1). We identified 2 pts with *VEGFA* and *KDR* (*VEGFR2*) mutations with corresponding VEGFR2 pathway activation (Table 1). The CORRECT trial led to the approval of regorafenib in mCRC pts with a 1.4-month OS (HR 0.77, P=0.0052) compared to placebo (24,26). Pts were not selected based on an ‘angiogenic’ biomarker to determine clinical response to regorafenib which may have shown benefit if the biomarker was predictive of response. Correlative studies in the CORRECT trial

investigated plasma angiogenesis proteins as potential biomarkers. Elevated level of TIE-1 was found to be associated with improved outcomes with regorafenib in terms of OS (27). Also, previous genotypic work correlated regorafenib treatment outcomes with single nucleotide polymorphisms in VEGF and VEGFR. The presence of VEGF-A rs2010963 was independently correlated with PFS and OS (HR: 0.49, 95% CI, 0.33–0.81 and HR: 0.52, 95% CI, 0.34–0.99), representing promise as a potential biomarker (26). Using NGS data to identify pts angiogenic targets (e.g., *PDGFRA*, *VEGFA*, *KDR*, *FLT1*, *FLT4*) and CMS4 (mesenchymal, stromal infiltration and increased angiogenesis) may provide insights in the regorafenib patient cohort in the CORRECT trial. Pts who develop toxicities to regorafenib do so in the first few weeks of initiating treatment, one could hypothesize that early toxicities manifest in tumors that are not biologically dependent on regorafenib. Identifying a selected population for TKI therapy may be clinically meaningful with an improved quality of life.

Discussion

Molecular subtyping coupled to a deeper understanding of cancer biology is clearly shifting the paradigm of cancer treatments. Biomarker approach to therapy has had some success as evident in metastatic breast cancer, lung cancer, gastric cancer and colon cancer enabling careful preselection of pts that benefit from targeted therapies (28-31). In 2015, elegant data reveals that MSI alone identifies pts in both CRC and non-CRC cohorts that may benefit from checkpoint blockade with anti-PD-1 monoclonal antibody therapy (6). Therefore, implementing the use of NGS in order to ascertain key genetic characteristics of tumors is instructive. Current understanding mandates a multimodality approach involving cytotoxic chemotherapy, targeted therapy and immune checkpoint therapy. However, considering a more favorable toxicity profile, numerous clinical trials have been designed to compare targeted molecular or immune therapy to standard of care cytotoxic therapy (32,33). Despite current advances (3,30), new therapies targeting angiogenesis (e.g., ziv-aflibercept, ramucirumab, regorafenib) have not moved the field significantly (26,34,35). Cytotoxic chemotherapy continues to add modestly in relapsed and refractory mCRC with the recent FDA approval of TAS-102, a novel oral combination of trifluridine, a thymidine-based nucleic acid analogue with a thymidine phosphorylase inhibitor, tipiracil (36).

Although in the relapsed and refractory setting of mCRC, NGS is being performed in academia and in community oncology clinics, a clear step wise systematic approach to when to order and how to interpret data is not established. We utilized NGS to test the hypothesis that dominant oncogene mutations do not always equate to the phenotype and an understanding of the underlying interactome in each patient provides clues to choosing the next best therapy, hopefully within a clinical trial setting. We highlight that the 'interactome' derived from NGS represent a meaningful tool to: (I) evaluate functional investigation of novel candidate molecular targets in the laboratory; (II) predict response and/or resistance to drug therapy; (III) guide rational drug combinations in relapsed and refractory mCRC pts.

Recently, four consensus molecular subtypes (CMS) for CRC was identified by an international consortium (8) based on six independent classification systems from 18 CRC data sets, including TCGA representing 4,151 patients. These include: CMS1 (14%), defined as MSI, CpG island methylator phenotype (CIMP) high, hypermutated,

associated with *BRAF* mutations and signaling pathways significant for immune infiltration and activation; CMS2 (37%), defined as canonical, with somatic copy number alterations (SCNA) and WNT and MYC pathway activation; CMS3 (13%), defined as metabolic with mixed MSI status, SCNA low, CIMP low, associated with *KRAS* mutations and pathways responsible for metabolic dysregulation; CMS4 (23%), SCNA high with pathways responsible for stromal infiltration, TGF- β activation, and angiogenesis. A mixed feature group reflected the remaining 13% of tumors was postulated to represent a transition phenotype (8). The data also reveals that aside from the association of *BRAF* mutations in CMS1 and *KRAS* mutations in CMS3, there was no isolated event or specific genetic alteration limited to each subtype. The development of *EGFR* ectodomain S492R mutations in *KRAS* WT pts treated with cetuximab therapy represents an example of this concept producing acquired resistance to biologic therapy (37). We present a redefined approach to mCRC pts using NGS plus pathway-network analysis within the CMS categories to test targeting aberrant biology in clinical trials (Figure 4).

Incorporation of CMS and NGS plus pathway-network analysis may better guide the management of pts with advanced mCRC. Our study represents a novel systematic approach to incorporating 'mutational signatures' into an 'interactome' in clinical decision making. This approach will aid in the identification of new targets that warrant further large scale prospective investigation, highlight potential biomarkers of response and resistance. In this paper we illustrate that dominant oncogene mutations do not always equate with oncogenic dependence and understanding cross-talk between activated signaling pathways and feedback loops in heavily pretreated relapsed and refractory mCRC pts is key to successful drug development. Our work is hypothesis generating for identifying novel targets and corresponding networks. NGS enhances elucidation of tumor clonal evolution and pathway-network analysis elaborating new and anticipated drug resistance mechanisms. The PROSPECT-C, reported that obtaining circulating tumor DNA of a patient with mCRC while on cetuximab therapy revealed a compensatory rise and fall in mutant *RAS* clones in response to the presence or absence of anti-EGFR therapy (38). As reported earlier within our cohort of pts, NGS data from new liver metastasis was obtained in a patient with *RAS* wild-type mCRC heavily pretreated with chemotherapy, anti-VEGF therapy and anti-EGFR therapy that subsequently developed coexistent

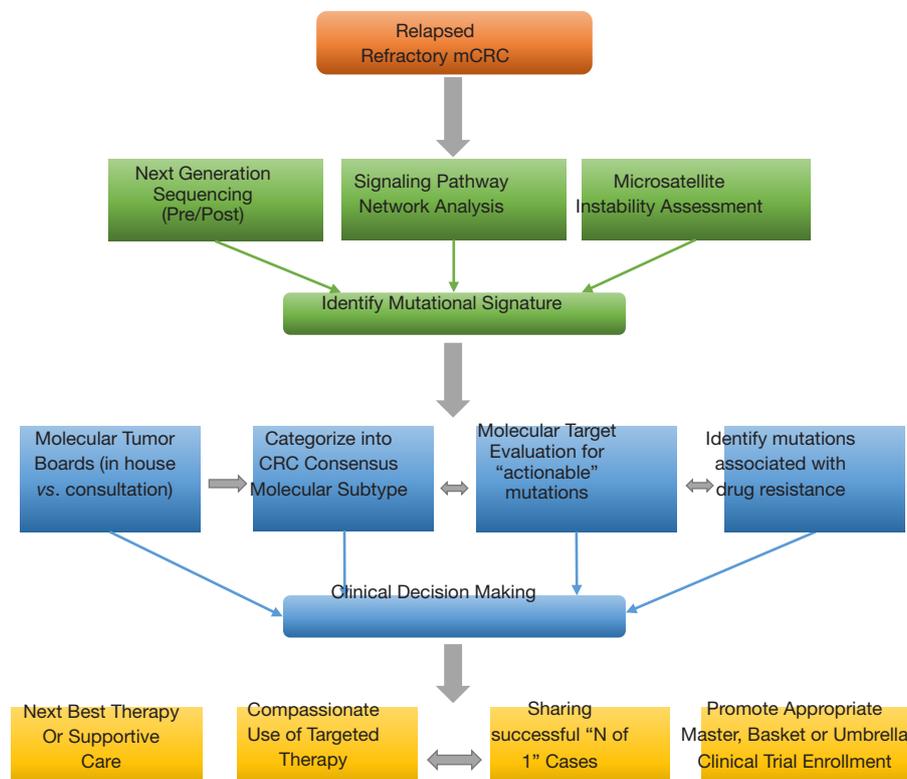


Figure 4 A management approach incorporating genomic data for every patient with relapsed and refractory mCRC. mCRC, metastatic colorectal cancer.

KRAS (Q61L) plus *NRAS* (Q61H) mutations. This showcases the “real-time” insight obtaining ‘pre/post’ NGS data, provides to document evidence of tumor evolution and facilitates a personalized approach to next best therapy in acquired genomic changes.

We propose an approach regarding the utilization of genomic data in the management of pts with mCRC (Figure 4). This approach represents an organized means to incorporate NGS and pathway-networks of each patient with mCRC furthering new research hypothesis, novel clinical trial design and enrollment using “N of 1” for reporting therapeutic success. This approach may result in a deeper understanding of the complex genomic landscape in advanced mCRC. Our approach (Figure 4) may aid in validation of this technology as a critical clinical decision making tool for advanced mCRC.

Conclusions

Expanded *RAS* mutational status in CRC is a clinically validated but very limited biomarker that guides current

therapeutic modalities. Emerging data indicate that *BRAF* mutation status is prognostic and predictive for response to anti-EGFR therapy (39) but is also limited. Although the use of NGS in relapsed and refractory mCRC is widespread in clinical practice, guidelines reflecting systematic incorporation of genomic data into daily patient care is in its infancy. Our experience in the utilization of NGS plus bioinformatics tools to identify signaling pathways representing a distinctive ‘mutational signature’ or ‘interactome’ for individual mCRC pts may allow for a tailored approach to therapy. The mutational signatures of thirty-two pts with relapsed and refractory mCRC identified *HER2*, *HER3*, *PDGFRA*, *ATM*, *ALK*, *ROS1*, *CDKN2A*, and *PIK3CA* as potential targets of focus and *HER2* and *FGFR* as unique activated feedback-escape pathways of interest for rational drug development. A focus on the ‘interactome’ highlights the biologic context of each patient’s mutational profile and represents a unique approach to laboratory investigation, enhancing drug development, directing next best therapy and appropriately triaging patients to enrollment on genomic clinical trials.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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