Title: Streamlining Detection of Fusion Genes in Colorectal Cancer: Having "Faith" in Precision Oncology in the (Tissue) "Agnostic" Era.

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Significant statement

The U.S. Food and Drug Administration recently granted tissue-agnostic approval for the first-in-class TRK inhibitor larotrectinib for patients whose tumors harbor fusions in neurotrophic receptor tyrosine kinases (NTRKs). These fusion genes have a frequency of less than 1% in unselected colorectal cancer patients. Using a multi-omics approach and a clinically annotated cohort of CRC patients, *Cocco and colleagues* showed that patients with sporadic, *RAS*/*BRAF* wild-type, mismatch repair deficient CRC tumors with MLH1 promoter methylation present fusions in kinase genes in 42% of cases, and suggested a diagnostic framework to improve the selection of patients eligible for gene fusion testing.

Commentary

Genomic instability represents a key hallmark of cancer. Indeed, the presence of microsatellite or chromosomal instability underpins cancer initiation and progression in many tumor types, including colorectal cancer (CRC) (1). In tumors with microsatellite instability (MSI), the presence of errors in short stretches of repeated nucleotides (microsatellites) is an epiphenomenon of defects in DNA mismatch repair (MMR) machinery genes (2). On the other hand, chromosomal rearrangements can lead to balanced or unbalanced structural changes in coding and non-coding areas of the genome, resulting in gene fusions that often act as oncogenic drivers (3). MSI occurs in approximately 2-3% of all cancers and approximately 3-5% of metastatic CRC (2). Gene fusions appear to be involved in about 16% of all cancers types, but targetable fusions involving kinases only occur in a minority of unselected CRC cases (3).

Recently, the U.S. Food and Drug Administration (FDA) granted two tissue-agnostic accelerated approvals for anti-cancer agents based on their remarkable clinical activity irrespective of tumor type: the PD-1 inhibitor pembrolizumab was granted extended approval to all patients with metastatic MSI-High (MSI-H) tumors in 2017; and the first-in-class TRK inhibitor larotrectinib was recently approved for patients whose tumors harbor fusions in neurotrophic receptor tyrosine kinase (NTRK) 1, 2 or 3 (4). Based on these ground-breaking findings, other tissue-agnostic drugs are currently in the pipeline; however, several hurdles remain. First, the frequency of some of these genomic abnormalities is relatively low in the general cancer population, thus the identification of potentially responsive patients through rapid and cost-effective diagnostic tests is a major challenge. Second, there is a cultural barrier between genomic-driven tissue-agnostic cancer medicine and the disease-oriented approach of oncologists. Even though the implementation of tumor molecular profiling boards in many institutions worldwide has contributed to improve patients' access to international basket trials, the identification of specific groups of patients who are most likely to benefit from tissue-agnostic drugs remains an unmet need.

In the current issue of *Cancer Research*, Cocco and colleagues characterized kinase fusions in patients with advanced CRC who underwent the MSK-IMPACT molecular testing at Memorial Sloan Kettering Cancer Center (MSKCC) over a period of approximately four years (5). Tumors were tested for MSI, *RAS*/*BRAF* mutations, MLH1 promoter methylation (MLH1p). Gene fusions were assayed and/or validated using a custom RNA-seq panel; a subset of cases also underwent genome-wide methylation profiling using the Illumina MethylationEPIC platform.

Overall, kinase gene fusions were identified in 0.9% of the 2314 CRC cases, confirming the low frequency of these genomic aberrations in an unselected CRC population. However, when multi-omics data were combined and gene fusions were put in context of other molecular features, the authors observed that the frequency of fusions was significantly higher in the MSI-H population (5%) compared to the microsatellite stable (MSS) group (0.4%; $p<0.001$). Fusions appeared more frequent in sporadic MMR-deficient MSI-H tumors with MLH1p and *RAS*/*BRAF* wild-type genotype (**5**). Based on these findings, the authors proposed a diagnostic flow-chart that would help to rationalise molecular testing for gene fusions in CRC (**5**). Indeed, the combined use of MSI and *RAS*/*BRAF* testing that is already routinely used in clinical practice, along with the determination of MLH1 promoter methylation status, might help to narrow down the number of CRC patients to be tested for gene fusions resulting in a more sustainable and cost/effective approach. Despite a few limitations related to the relatively small cohort and the fact that none of the patients with kinase fusions were in fact treated with driver-specific tyrosine kinase inhibitors, the study undoubtedly offers a new angle for the implementation of precision oncology in routine clinical practice.

In line with the data reported by Cocco and colleagues (**5**), Sato and colleagues analyzed nearly 3000 CRC cases from Japan and described remarkably similar results, with tumors harboring fusions in kinase genes being enriched in sporadic MSI-H, MLH1p, *RAS*/*BRAF* wild-type CRC cases (**6**): 55% of sporadic CRC patients with MMR deficiency, MLH1p, and lack of mutations in the RAS/BRAF pathway harbored fusions genes. When analysing MSS cases, the two cohorts showed almost identical frequency in fusion genes (0.4% in the MSKCC vs 0.5% in the Japanese cohort) (**5**,**6**).

Consistent with these data, previous reports (**7**) have suggested an association between fusions in targetable kinase genes and MSI-H and *RAS*/*BRAF* wild-type status, and also highlighted a potential correlation between fusion genes and sidedness, thus providing one more clinical variable for the decisional algorithm. Cocco and colleagues reported that approximately 70% of patients with gene fusions had proximal (i.e. right sided) primary cancers (**5**), and these data seem to align well with those presented by Pietrantonio and colleagues who reported 80% of patients with fusions being right sided (**7**). These correlations between occurrence of fusion genes and sidedness are based on small numbers, thus require further validation before final conclusions can be drawn; however, if confirmed, they may offer a further clinical parameter to be incorporated in the decisional tree for the selection of patients' eligibility for gene fusion testing. Furthermore, any association between right sided tumors and increased frequency in fusions involving kinases might explain some of the cases of inherent resistance to epidermal growth factor receptor

(EGFR) monoclonal antibodies in *RAS*/*BRAF* wild-type right sided primary CRC and offer potential therapeutic options for this subset of CRC patients. Compared to the study by Pietrantonio and colleagues (7), the lack of detailed information on patient outcomes in the MSKCC study (5) precluded any analysis on the prognostic role of gene fusions among MSI-H patients. However, it is intriguing to believe that gene fusions might contribute to the poor prognosis of metastatic *BRAF* wild-type MSI-H CRCs as this may justify the lack of significance in the interaction test between MSI-H status and *BRAF* mutations in the pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies (8).

BRAF V600E mutations have been linked to the acquisition of the GpG Island Methylator phenotype (CIMP) in sporadic MSI-H CRC. Indeed, upon activation of the BRAF/MEK/ERK signalling pathway, the transcriptional repressor MAFG is phosphorylated, and along with corepressor partner proteins such as BACH1, CHD8, and the DNA methyltransferase DNMT3B, binds to the MLH1 promoter and other CIMP genes causing hyper-methylation and transcriptional silencing (9). Given the mutual exclusivity between fusions in kinase genes and activating *BRAF* mutations reported in the current and other similar studies (5-7), Cocco and colleagues (5) hypothesized that fusion genes might be responsible for inducing the CIMP phenotype in *BRAF* wild-type CRC; however, given that different onco-proteins might trigger the assembly of distinct repressor complexes on common promoters (9), this hypothesis will require further experimental validation using pre-clinical models.

Although few cell lines and patient-derived xenografts from CRC patients with kinase genes fusions are already available, these fall short in capturing inter- and intra-patient heterogeneity. From this prospective, refining the selection criteria for testing kinase gene fusions might translate into a more accurate selection of patients for molecular screening, thus enabling bio-banking efforts of patient-based models such as organoids (10) mimicking uncommon genetic drivers. These models might prove critical for forward and reverse translation in order to understand molecular mechanisms and overcome primary or acquired resistance.

Whether the diagnostic framework proposed by Cocco and colleagues could be extrapolated to other cancer types remains to be seen. Nonetheless, this study highlights the paramount importance of integrating multi-omics data with detailed clinical annotation in defining niches of patients with targetable driver genes and, as such, likely to benefit from precision medicine approaches. Although tissue-agnostic treatments are based on mechanisms of action rather than tissue specificity (4), an in-depth understanding of the molecular portraits of different tumor types is pivotal to identify sub-groups of patients enriched for certain

genomic drivers, thus allowing resource prioritization and timely and cost/effective delivery of genomic characterization in the clinic.

The results reported by Cocco and colleagues (**5**) widen the therapeutic horizon for some *RAS*/*BRAF* wild-type MSI-H colorectal cancers as these patients may benefit from both FDA-approved tissue-agnostic treatments. However, it is worth mentioning that these patients represent a relatively small proportion of all metastatic CRC, and future efforts should continue to investigate better ways to identify fusion genes in MSS patients. Approximately one in two hundred MSS CRC patients harbors targetable gene fusions (**5-7**), and 97% of metastatic CRC cases are in fact microsatellite stable (**2**). When these numbers are looked in the context of incidence of new metastatic CRC cases per year, they unveil the presence of a significant proportion of CRC patients that might gain a clear benefit from targeted therapies with kinase inhibitors and, as such, cannot be neglected.

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