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## Rational testing for gene fusion in colorectal cancer: MSI and *RAS-BRAF* wild-type metastatic colorectal cancer as target population for systematic screening

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**Abstract:**

Gene fusions provide access to new therapeutic opportunities for patients treated for a colorectal cancer (CRC). However, they do not exceed 1% of patients. A better identification of patients in whom gene fusions are highly prevalent is a major issue in a therapeutic and medico-economics perspective. This study assesses the rates of gene fusions in CRC patients with MSI/*RAS-BRAF*<sup>WT</sup> in our routine practice detected with a commercially available NGS-based fusion panel. Among the 130 MSI CRC tumors, 43 (33%) were *KRAS-NRAS-BRAF*<sup>WT</sup>. A gene fusion was detected in 7 (25.9%) of the 27 MSI/*RAS-BRAF*<sup>WT</sup> samples which had RNA suitable for analysis after quality control. These fusions involved mainly *NTRK1/3* (n=5), as well as *ALK* (n=1) and *BRAF* (n=1). In the present study, we confirm that patients with MSI/*RAS-BRAF*<sup>WT</sup> CRCs represent a subpopulation in which targetable gene fusions are overrepresented. Our results support the use of a two-step algorithm for molecular screening, in which metastatic CRC patients would have routine MSI and *RAS/BRAF* testing, and then only those with MSI/*RAS-BRAF*<sup>WT</sup> would be screened with dedicated NGS RNA panel for gene fusions.

**Keywords:** gene fusion, colorectal cancer, targeted therapy, medico-economics

## Introduction

Gene fusions have been described since several years in colorectal cancer (CRC). Neurotrophic tyrosine receptor kinase (*NTRK*) gene rearrangements were the first to be discovered in 1986 [1] and more than 80 fusion partner genes have been described thereafter [2] [3]. Further, fusions involving *ALK*, *ROS1*, *RET* [4], *BRAF*, *FGFR* [5], *ERBB2* [6], and *NRG1* [7] were identified.

In the recent years, these alterations have turned into therapeutic opportunities for patients with the development of new targeted therapies. Among them, larotrectinib and entrectinib showed clinical activity in a broad spectrum of adult and pediatric solid tumors harboring *NTRK* fusions [8] [9], leading to a tumor-agnostic approval by the FDA and EMA. These inhibitors are available in current practice for patients with chemo refractory metastatic CRC with *NTRK* fusions (< 1%). Other inhibitors, targeting *ALK* or *RET* fusions (initially developed for non-small cell lung cancers) and *FGFR* rearrangements (for cholangiocarcinoma), are also available for metastatic CRC patients through basket clinical trials.

However, in unselected metastatic CRC patients (“all comers”), gene fusion assessment has a low diagnostic yield. Indeed, each individual fusion represents less than 0.1% to 0.5 % of CRC, and all combined together they do not exceed 1% of patients [5]. Moreover, for optimal sensitivity, gene fusion detection relies on RNA (rather than DNA) sequencing, thus requiring specific extraction and next-generation sequencing (NGS) panels. Given the costs of these techniques and the high incidence of CRC, they cannot be systematically deployed in all patients. Therefore, a better selection of patients that could benefit from gene fusion testing is a major cost-utility issue.

Recent studies reported that CRC harboring microsatellite instability (MSI) (particularly, sporadic cases related to *MLH1* promoter methylation), and even more those combining MSI and absence of mutations in *RAS-BRAF* (*RAS-BRAF*<sup>WT</sup>), were enriched in gene fusions [5] [10] [11], and particularly *RET* fusions [12]. This suggests that patients with MSI/*RAS-BRAF*<sup>WT</sup> tumors could be an interesting subgroup for gene fusion testing. The aim of this study was to further assess the rates of gene fusions in CRC patients with MSI/*RAS-BRAF*<sup>WT</sup> in our routine practice.

## **Material and methods**

### *Tumor samples*

One hundred and thirty tumors from 129 patients with MSI CRC from four French hospitals were retrieved and analyzed. Two tumors were from a same patient who underwent right and left colectomy for *de novo* cancers 13 months apart. Clinico-pathological data were retrospectively collected. The study was conducted in accordance with the ethics principles of the Declaration of Helsinki and General Data Protection Regulation (GDPR). According to French regulations, this study did not need an informed consent. Patients were informed of the study by each investigator and did not express opposition.

### *DNA extraction and gene mutation detection*

Tumor DNA of the 129 patients was extracted from formalin-attached tissues incorporated into paraffin (FFPE). The tumor samples were digested using proteinase K in an incubator at 56°C for three days. The DNA was isolated with the Nucleospin® 8 Tissue kit (Macherey-Nagel, GmbH & Co. KG, Germany). Mutations of *KRAS*, *NRAS* and *BRAF* were analyzed by High Resolution Melting (HRM).

### *RNA extraction and gene fusion detection*

Tumor RNA of *KRAS-NRAS-BRAF*<sup>WT</sup> patients were isolated from FFPE with the *High Pure FFPE RNA Micro* Kit (Roche Diagnostics, Germany) according to the manufacturer's instruction. Analyses of RNA quantity were performed using NanoDrop.

For the detection of gene fusions, the Archer FusionPlex® CTL panel (Archerdx Boulder, CO, USA) was used according to manufacturer's instructions. This dedicated NGS panel is designed to target 17 genes involved in fusions that are commonly observed in solid tumors (i.e.: *ALK*, *AXL*, *BRAF*, *CCND1*, *FGFR1*, *FGFR2*, *FGFR3*, *MET*, *NRG1*, *NTRK1*, *NTRK2*, *NTRK3*, *PPARG*, *RAF1*, *RET*, *ROS1* and *THADA*).

200 ng of RNA was enriched and prepared libraries were sequenced on a MiSeq (Illumina). The Archer Analysis software (Archerdx) was used for data analysis and gene fusion detection.

When the quantity and quality of RNA do not allow to conclude (RNA degraded, quality control status failure...), sample was considered non-contributory (NC).

## Results

Among the 130 MSI CRC tumors, 43 (33%) were *KRAS-NRAS-BRAF*<sup>WT</sup> and 87 (67%) harbored a *KRAS*, *NRAS* or *BRAF* mutation (**Figure 1**).

A total of 27 MSI/*RAS-BRAF*<sup>WT</sup> samples (62,8%) had RNA suitable for analysis after quality control. Of them, a gene fusion was detected in 7 (25.9%). These fusions involved mainly *NTRK1/3* (n=5), as well as *ALK* (n=1) and *BRAF* (n=1). Patient clinical characteristics are presented in **table 1**. A targeted therapy could have been proposed to 5 of 6 these patients (*NTRK* and *ALK* fusions) [13].

## Discussion

A better identification of patients in whom tumoral gene fusions are highly prevalent is a major issue in a therapeutic and medico-economics perspective. In the present study, we confirm that patients with MSI/*RAS-BRAF*<sup>WT</sup> CRC, represent a subpopulation in which gene fusion are overrepresented, reaching 25.9%. All patients had MLH1/PMS2 loss. As a comparison, gene fusions have been reported in only 5% of CRC patients with MSI/dMMR (and were mutually exclusive with *BRAF*<sup>V600E</sup> and *RAS* hotspot mutations in this population) and in less than 1% of unselected CRC [5].

Our results are consistent with those from three recently published studies showing an enrichment in gene fusions in MSI/*RAS-BRAF*<sup>WT</sup> CRC [5] [10] [11] (**Table 2**). In contrast, we did not confirm the high prevalence of *RET* fusion (26%) reported by Pietrantonio et al. [12]. The small sample size of the studies can account for the variability of percentages of gene fusions that are detected.

An interesting point is the enrichment of fusions in MSI CRC related to MLH1 loss, and particularly those with hypermethylation of *MLH1* promoter (i.e. so called sporadic or spontaneous MSI)

suggested by several authors [5] [10] [11]. We did not explore this point in our work. In about 20%-30% of cases, *BRAF* V600E mutation is associated with sporadic MSI CRC. The mechanistic basis for the relationship between *BRAF* V600E, genome-wide hypermethylation, and MSI has been proposed by Fang et al. [14], who showed that *BRAF* V600E mutations in CRC induce CpG island hypermethylation including *MLH1* promoter. The strong relationship between RTK-RAS fusions and *MLH1* promoter hypermethylation suggests that fusions may induce a similar phenomenon. Indeed, results from Cocco et al. [5], using methylation array studies, showed that *BRAF* V600E mutant and tyrosine kinase receptor (RTK) fusion positive CRC have similar genomic CpG methylation patterns, even after exclusion of data from the *MLH1* promoter CpG loci. Further research, including functional studies, are needed to elucidate the molecular mechanisms involved in this relationship between RTK fusions and *MLH1* promoter hypermethylation.

Bocciarelli *et al.* [10] showed that simple techniques, i.e. immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH), could be used with low rates of false positive (1 of 13 cases). However, these techniques have limited sensitivity for some fusions (e.g. *FGFR2*) and require a gene-by-gene testing approach, as opposed to NGS panels allowing the simultaneous screening of several rare gene fusions. Furthermore, Wang *et al.* [11] showed a better sensibility of RNA NGS over DNA NGS (4 additional among 22 fusions were only detected by RNA NGS). Overall, RNA NGS panels display the best diagnostic performance for gene fusion testing in solid tumors.

As an apparent paradox, MSI CRC associated with fusions, although bearing a high mutational burden, do not carry other targetable genomic alterations. Gene fusions are important oncogenic drivers, which could explain the absence of other genomic alterations. Particularly, gene fusions identified in CRC affect the tyrosine kinase receptor-RAS (RTK-RAS) pathway. Aberrant activation of RTK-RAS signaling pathway is well-recognized as key and early molecular event in CRC tumorigenesis. RTK-RAS activation is generally mediated by *KRAS* or *BRAF* oncogenic mutations (both found in 20%-30% of metastatic MSI CRC [15]), which are described as mutually exclusive. It is rational to assume that as one oncogenic mutation is sufficient to activate in the RTK-RAS pathway, no other redundant event in

this pathway is further selected. It has been reported that, similar to *KRAS* and *BRAF* mutations, gene fusions are one major mechanism of RTK-RAS oncogenic activation and are mutually exclusive with other oncogenic mutations [3] [11].

Our observation of high rate of gene fusions in patients with MSI/*RAS-BRAF*<sup>WT</sup> CRC ties in with a more global concept in digestive oncology, according to which when a strong oncogenic driver such as *KRAS* or *BRAF* mutation is present, it is extremely unlikely to identify another targetable alteration. For example, in pancreatic adenocarcinoma, more than 90% of which carry a *KRAS* activating mutation, fusion transcripts are detected almost exclusively in tumors without a *KRAS* mutation, making it possible to propose NGS RNA panel only to *KRAS* wild-type patients [16]. A similar two-step strategy could thus be proposed in CRC, starting with the search for *RAS/BRAF* mutations and MSI status, to select the patients most likely to benefit from RNA, although the costs of these analyzes are doomed to decrease over the years.

Our study has several limitations. These include the rarity of kinase fusions in CRC resulting in a relatively small cohort and the fact that none of the patients received a tyrosine kinase inhibitor and had available response data. Larger observational and interventional studies are thus warranted.

#### **Conclusions:**

Our results support the use of a two-step algorithm for molecular screening, in which metastatic CRC patients would have routine MSI (4-5% at the metastatic stage) and *RAS/BRAF* testing, and then only those with MSI/*RAS-BRAF*<sup>WT</sup> (1-2% of the totality of the metastatic CRC) would be screened with dedicated NGS RNA panel for gene fusions. This rational approach would allow conciliating the objective to not miss a therapeutic opportunity for these patients with resources and cost optimization.

**Declaration of Interest statement:** none declared

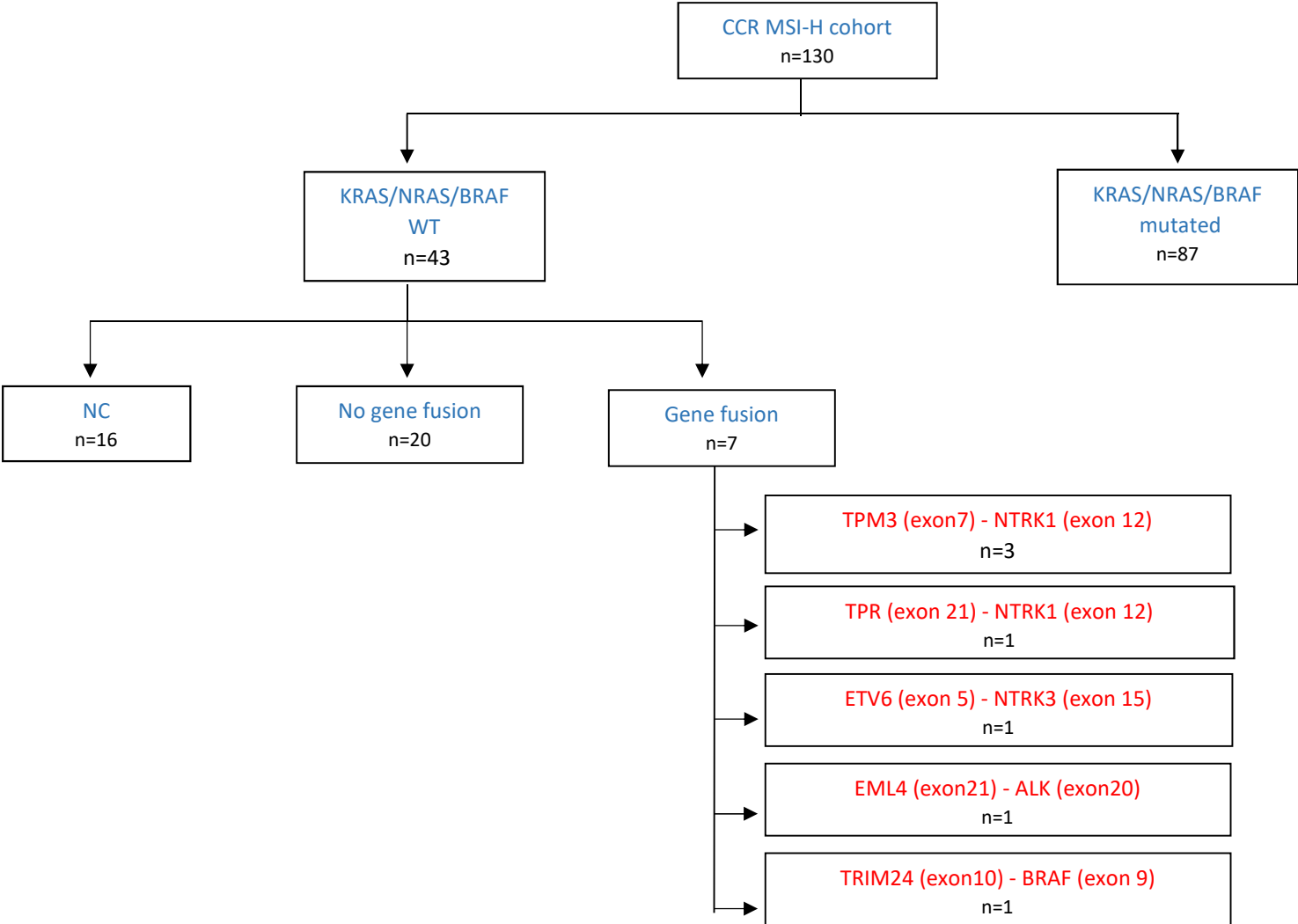


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Figure 1: Gene fusion detection



**Table 1: Patient clinical characteristics**

N°	sex / age(years)	tumor localization	tumor stage (WHO TNM 8th Ed)	Number of colected/metastatic lymph nodes	MSI	Histological type (WHO 5th Ed)	Gene Fusion
1	H / 64	caecum	pT3N0	15 / 0	loss of MLH1/PMS2	adenocarcinoma, 30% mucinous	ETV6 (exon 5)-NTRK3 (exon 15)
2	H / 73	transverse colon	pT4aN0	24 / 0	loss of MLH1/PMS2	poorly cohesive carcinoma	TPR (exon 21)-NTRK1 (exon 12)
3	H / 59	caecum	pT4aN1a	22 / 1	loss of MLH1/PMS2	poorly cohesive carcinoma	TPM3 (exon7) - NTRK1 (exon 12)
4	F / 84	right colon	pT4aN2b	33 / 10	loss of MLH1/PMS2	poorly cohesive carcinoma	TPM3 (exon7) - NTRK1 (exon 12)
5	F / 85	rectum	pT3N2	17 / 3	loss of MLH1/PMS2	mucinous adenocarcinoma	TPM3 (exon7) - NTRK1 (exon 12)
6	F/72	right colon	pT2N0	16/0	loss of MLH1/PMS2	Adenocarcinoma	EML4 (exon21)-ALK(exon20)
7	F/73	transverse colon	pT4aN2aM1c	24/4	loss of MLH1/PMS2	mucinous adenocarcinoma	TRIM24 (exon10)-BRAF(exon 9)

\*4 and 5 are samples from a same patient who underwent right and left colectomy 13 months apart.

**Table 2: Studies assessing gene fusion in MSI/RAS-BRAF<sup>WT</sup> colorectal cancers.**

Cohort	Method	N	RAS/RAF <sup>WT</sup> N(%)	Gene fusion	
MSI-H cohort	Archer Panel	130	43 (33)	<i>NTRK</i> (n=5) <i>ALK</i> (n=1) <i>BRAF</i> (n=1)	Present study
"All comers" Colorectal cancers	MSK-IMPACT +/- Archer Panel	2314 Including 230 (MSI/dMMR)	1051(45) 71 (31) in the MSI/dMM group	<i>NTRK</i> (n=8) <i>BRAF</i> (n=5) <i>RET</i> (n=4) <i>FGFR</i> (n=2) <i>ROS1</i> (n=1) <i>ALK</i> (n=1)	Cocco <i>et al.</i> [5]
MLH1 deficient	IHC +/- FISH + Archer Panel	84	18 (21)	<i>NTRK</i> (n=8) <i>BRAF</i> (n=2) <i>ALK</i> (n=1) <i>RET</i> (n=1)	Bocciarelli <i>et al.</i> [10]
dMMR	RNA NGS + DNA NGS	193	39 (20)	<i>NTRK</i> (n=14) <i>ALK</i> (n=3) <i>RET</i> (n=2) <i>MET</i> (n=1) <i>BRAF</i> (n=1) <i>MAPK1</i> (n=1)	Wang <i>et al.</i> [11]