ALK, ROS1, and NTRK Rearrangements in Metastatic Colorectal Cancer

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ALK, *ROS1* and *NTRK* rearrangements define a new subtype of metastatic colorectal cancer

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Running head: *ALK*, *ROS1* and *NTRK* rearranged metastatic colorectal cancer

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Abstract

Background: *ALK, ROS1* and *NTRK* fusions occur in 0.2%-2.4% of colorectal cancers. Pioneer cases of mCRC patients bearing rearrangements who benefited from anti-ALK, ROS, TrkA-B-C therapies were reported.

Methods: Clinical features, molecular characteristics and outcome of 27 mCRC patients bearing *ALK*, *ROS1*, *NTRK* rearranged tumors were compared with those of a cohort of 319 patients not bearing rearrangements. Deep molecular and immunophenotypic characterizations of rearranged cases, including those described in the TCGA database, were performed.

Results: Closely recalling the "*BRAF* history", *ALK*, *ROS1* and *NTRK* rearrangements more frequently occurred in elderly patients (p=0.024) with right-sided (p<0.001) and node-spreading (p=0.03), *RAS* wild-type (p<0.001), MSI-high (p<0.001) cancers. All patients bearing *ALK, ROS1,* and *NTRK* fusions had shorter overall survival (15.6 months) than negative patients (33.7 months), both in the univariate (HR 2.17, 95%CI 1.03-4.57; p<0.001) and multivariate models (HR 2.78, 95%CI 1.27-6.07; p=0.011). All four evaluable patients with rearrangements showed primary resistance to anti-EGFRs. Frequent association with potentially targetable *RNF43* mutations was observed in MSI-high rearranged tumors.

Conclusion: *ALK*, *ROS1*, *NTRK* rearrangements define a new rare subtype of mCRC with extremely poor prognosis. Primary tumor site, MSI-high, *RAS* and *BRAF* status may help to identify patients bearing these alterations. While sensitivity to available treatments is limited, targeted strategies inhibiting ALK, ROS and TrkA-B-C provided encouraging results.

Genomic translocations leading to the constitutive activation of receptor tyrosine kinases (RTKs) play a crucial role in tumorigenesis across different malignancies, including colorectal cancer (CRC) [1,2]. RTK fusions involving *ALK, ROS1,* and *NTRK1-2-3 (NTRK)* occur in 0.2%-2.4% of CRCs [3,4], and may represent new targets for therapeutic intervention [5-17]. Addiction to kinase suppression or pharmacological inhibition has been reported in CRC preclinical models bearing RTK fusions, including the *TPM3-NTRK1* rearranged KM12 cell line [18], the *ALK* rearranged cell line C10 [19], patient-derived primary cell lines [10] and patient-derived xenografts [20]. So far, a single heavily pre-treated metastatic CRC (mCRC) patient whose tumor bore an *LMNA-NTRK1* fusion was treated with entrectinib, an oral selective inhibitor of ALK, ROS1, and TrkA-B-C (the protein products of the *NTRK1-2-3* genes, respectively), with clinical benefit [15]. Another mCRC patient whose tumor harbored *STRN-ALK* fusion received the oral ALK inhibitor ceritinib and achieved response [16], and a patient with a *CAD-ALK* rearrangement responded to entrectinib [6].

Despite these pioneer case reports, it has not been clearly established whether *ALK, ROS1,* or *NTRK* rearranged tumors represent a distinct, although rare, disease subtype that should be detected early in order to adopt a tailored management strategy that may include targeted treatments.

Although a few reports have described the occurrence of *ALK*, *ROS1* and *NTRK* fusions in CRC (Supplementary Table 1), there is still limited knowledge about clinical and pathological characteristics, prognosis and sensitivity of these tumors to available treatments including anti-EGFR monoclonal antibodies (MoAbs) such as cetuximab and panitumumab. Similarly, except for some preclinical reports [11,19], comprehensive molecular and functional data to clarify whether these alterations confer oncogene addiction and to suggest perspectives on optimal treatment strategies are not available yet.

We therefore carried out a global effort aimed at characterizing the molecular and clinical landscape of *ALK*, *ROS1* and *NTRK* rearranged mCRCs. Even though a broader list of gene fusions has been described in CRC, including those affecting *RET*, *HER2* and *BRAF* [2,8,22,23], we specifically focused

on mCRC with *ALK, ROS1* and *NTRK* rearrangements since their phylogeny is closely related and they are frequently grouped as targets of newly developed agents such as entrectinib [24].

Methods

Study design and participants

In the clinical step (Figure 1), the cohort of 319 *ALK*, *ROS1* and *NTRK* negative cases included patients screened for Ignyta's phase 1 program at: Samsung Medical Center (SMC), Seoul, South Korea (n=209); Azienda Ospedaliero-Universitaria Pisana (AOUP), Pisa, Italy (n=79); Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy (n=31). The population of 27 *ALK*, *ROS1*, *NTRK* rearranged mCRCs included patients collected at: Foundation Medicine Inc. (FMI), Cambridge, Massachusetts (n=12); Samsung Medical Center (SMC), Seoul, South Korea (n=4); Memorial Sloan Kettering Cancer Center (MSKCC), NYC, New York (n=3); Austin Health, Heidelberg, Australia (n=3) on behalf of MAX trial Investigators; Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy (n=2); Niguarda Cancer Center (NCC), Milan, Italy (n=2); University Hospital Gasthuisberg (UHG), Leuven, Belgium (n=1). Molecular screening methods are detailed as Supplementary Methods and summarized in Figure 1. Study participants signed a written informed consent and the study was approved by the Institutional Review Board of INT, Milan.

Statistical analysis

We investigated the association of *ALK*, *ROS1* and *NTRK* rearrangements with the following variables collected at the diagnosis of mCRC: age, gender, ECOG performance status (0, ≥1), primary tumor location (right colon, left colon, rectum), primary tumor resection, mucinous histology, time to metastases (synchronous, metachronous), number of metastatic sites (1, >1), metastatic sites (lung, lymph nodes, liver, peritoneum), *RAS* and *BRAF* status (mutated, wild-type),

MMR status (proficient, deficient). Fisher's exact test, χ^2 test or Mann-Whitney tests were used when appropriate to assess the associations of the *ALK, ROS1, NTRK* rearrangements with investigated characteristics. Statistical significance was set at p=0.05 for a bilateral test.

We investigated the impact of *ALK*, *ROS1* and *NTRK* rearrangements on overall survival (OS), defined as the time from diagnosis of metastatic disease to death or last follow up for alive patients. OS analysis was determined according to the Kaplan-Meier method and survival curves were compared using the log-rank test. The correlation of *ALK, ROS1, NTRK* status and clinicopathological characteristics with OS was assessed in univariate analysis. In order to minimize the bias of multiple comparisons, according to the false discovery rate correction, statistical significance was set at p=0.009 for a bilateral test. Cox proportional hazard model was adopted in the multivariate analysis, including as covariates variables correlated with survival with p<0.1 in the univariate analyses. Hazards' proportionality was assumed.

All analyses were carried out by means of Prism 7 for Mac OS X v7.0.

Translational analyses

As shown in Figure 1 and Supplementary Methods, NGS data were obtained through 3 different panels: FMI panel in 15 cases, Minerva panel (Ignyta Inc.®) in 11 cases, MSK-IMPACT panel in 1 case. The association of individual samples with the type of translocation identified and NGS panel is shown in Supplementary Table 2. Finally, analysis in silico from TCGA data was performed (Supplementary Methods).

Results

Study population

Based on a systematic literature review, we identified 24 published cases of *ALK, ROS1* or *NTRK* rearranged CRCs (Supplementary Table 1). Nineteen were staged as metastatic, and informative medical records were retrieved for fifteen of them. Taking advantage of screening programs

worldwide, we were able to identify 12 additional cases. Therefore, the final population consisted of 27 *ALK*, *ROS1*, *NTRK* rearranged mCRCs (Figure 1; Supplementary Table 2) including a newly described *SCYL3-NTRK1* fusion (Supplementary Figure 1). We compared the clinical and pathological features of *ALK, ROS1,* and *NTRK* rearranged mCRCs with a cohort of *ALK*, *ROS1*, and *NTRK* negative patients (n=319), screened for phase 1 studies at three Institutions (Figure 1). The overall incidence of *ALK*, *ROS1*, or *NTRK* rearrangements at these Institutions was 1,5% (5 out of 324 screened samples).

Clinical and pathological features of ALK, ROS1 and NTRK rearranged mCRC

As shown in Table 1, rearrangements were more frequent in older patients (p=0.024) with right-sided tumors (80.0% vs 30.0%; p<0.001), and spread more frequently to lymph nodes (45.8% vs 24.7%; p= 0.030) and less frequently to the liver (41.7% vs 65.5%; p=0.026). Additionally, although only 50% of patients in the control group had available information on MSI status, a higher percentage of tumors bearing rearrangements were MSI-high (48.1% vs 8.1%; p<0.001).

Of note, *RAS* mutations were much less frequent in rearranged than in other tumors (7.4% vs 48.3%; p<0.001). Only one (3.7%) rearranged sample showed the co-occurrence of *SLC34A2-ROS1* fusion and BRAF V600E mutation. Overall, right-sided primary location, *RAS* wild-type and MSI-high status, in addition to female gender, were particularly associated with *NTRK* rearrangements. Notably, patients with right-sided, *RAS* and *BRAF* wild-type, MSI-high mCRCs had 54- and 453-fold higher chances of harboring *ALK, ROS1, or NTRK* rearrangements (OR=54.0, 95% CI: 13.3-219.1; p<0.001) or specifically *NTRK* rearrangements (OR=453.0, 95% CI: 67.2-3053.4; p<0.001), respectively. These four easy to collect characteristics (primary tumor site, MSI, *RAS* and *BRAF* status) enable identification of patients bearing an *ALK, ROS1,* or *NTRK* rearrangement with positive and negative predictive values of 75% and 95%. The positive and negative predictive values with specific regard to *NTRK* rearrangements were 75% and 99%.

Molecular features of ALK, ROS1 and NTRK rearranged CRC

Molecular reports from next-generation sequencing DNA analyses performed on rearranged cases were retrieved (Figure 1). Additionally, molecularly annotated genomic variants from seven CRC samples harboring *ALK* or *NTRK3* fusions (Supplementary Figure 2 and 3) in the TCGA database were gathered. First, we focused on the subset of genes previously reported as the most frequently mutated in CRCs (Figure 2A) [23] . In line with previous reports regarding MSI-high BRAF mutated CRC [24-26], MSI-high rearranged tumors were enriched for alterations affecting *RNF43* (64.7% vs 5.9%; p=0.0004 Fisher's exact test), most of which were frameshift changes affecting glycine 659, which lies within a mononucleotide repeat (Figure 2A).

A low prevalence of *RAS*/*BRAF* mutations, also accounting for MSI-high status (Figure 2B), was reported. Only one MSS rearranged tumor displayed a BRAF V600E mutation, while two MSI-high rearranged mCRC samples carried *BRAF* alterations (I371M and K475R) of unknown significance and two MSS rearranged CRCs showed a well-established oncogenic variant (G469A), and an alteration (D594H) that impairs BRAF kinase activity but paradoxically activates MEK and ERK through transactivation of CRAF, respectively. The prevalence of *PIK3CA* mutations in CRCs carrying rearrangements (12.1%) did not significantly differ from what reported in unselected colorectal tumors [23].

An explorative analysis of selected genes implicated in immune-escape mechanisms [27] was conducted by retrieving the transcriptomic profiles of the seven rearranged samples for which RNA seq data was available from the TCGA and these were compared with non rearranged MSI-high CRC samples also from TCGA (Figure 2C). Although the analysis suggested that the presence of rearrangements did not impact the typical MSI-high phenotype represented by the upregulation of immunoinhibitory molecules [27], the small number of samples limits the power of this observation.

Prognostic impact of ALK, ROS1 and NTRK rearrangements in mCRC

Finally, we explored the clinical impact of *ALK, ROS1* and *NTRK* rearrangements in the metastatic setting (TCGA samples were excluded from survival analyses, since they were mostly found in earlier disease stages and had incomplete follow-up data). When looking at OS results, at a median

follow-up of 28.5 months [95%CI 23.8-36.9], patients bearing *ALK, ROS1* or *NTRK* rearranged tumors had poor prognosis when compared with rearrangement negative tumors (median OS: 15.6 [95%CI 10.0-20.4] versus 33.7 [95%CI 28.3-42.1] months; HR for death: 2.17, 95% CI 1.03-4.57; p<0.001) (Figure 3A). When applying the false discovery rate correction, the association of *ALK*, *ROS1* and *NTRK* rearrangements with OS was still statistically significant (p<0.005). In the multivariable model (Table 2) including other covariates associated with OS with p<0.1 (age, primary tumor location, primary resection, *BRAF* mutation and MSI status), the presence of gene rearrangements was still associated with shorter OS [HR for death: 2.33, 95% CI 1.10-4.95; p=0.020]. Notably, patients with *ALK, ROS1* or *NTRK* rearranged tumors had short OS independently from MSI status (Figure 3B). In fact, median OS was 17.0 (95% CI 10.0-31.4) months for patients with MSS rearranged tumors and 15.6 (95% CI 10.0-20.4) months for MSI-high ones. Moreover, the poor prognostic impact of gene rearrangements was independent of primary tumor location: both in right- and left-sided tumors patients bearing rearrangements had shorter OS than those with negative tumors (Supplementary Figure 4).

Therapeutic implications of ALK, ROS1 and NTRK rearrangements in mCRC

All the patients with rearranged tumors that were treated with cetuximab or panitumumab (N=4) experienced disease progression as best response during the treatment with anti-EGFR agents (Supplementary Methods; Supplementary Figure 5).

One patient with *EML4-ALK* rearrangement and MSI-high tumor received single agent anti-PD-1 treatment with nivolumab and achieved a durable response (Supplementary Figure 5). Notably, the IHC staining of this tumor revealed intense staining for CD4, CD8, CD68 and especially PDL-1, with an abundant intra and extratumoral lymphocytic infiltration (Supplementary Figure 6).

Discussion

Here we showed that *ALK, ROS1* and *NTRK* rearrangements identify an uncommon CRC molecular subtype with specific clinical, pathological and molecular features. The investigated fusions (and particularly those affecting *NTRK*) were more frequent in elderly females with right-sided tumors, spreading to extra-regional lymph nodes. However, the most clinically relevant association was found with MSI-high and *RAS* wild-type status, which are two relevant and commonly used biomarkers for patient selection for immunotherapy and anti-EGFRs, respectively. This type of clinical and molecular associations resemble very closely what observed for codon 600 *BRAF* mutations and, interestingly, *BRAF* V600 mutations and gene fusions were almost invariably mutually exclusive. Since MSI-high status is reported in less than 5% of mCRCs [28], the frequency of MSI-high rearranged tumors is unexpectedly high (48.1%), even considering the right-sided location [29]. The frequency of MSI-high status in *ALK, ROS1* and *NTRK* rearranged tumors seems similar or even higher than in BRAF V600E mutated mCRCs, where it reaches 30-35% [24,28]. While the association between right-sided tumors, MSI-high and *BRAF* mutations is well established, we report for the first time a strong association with right-sided tumor location and MSI-high status also for gene fusions. Of note, while frame-shift mutations occurring in MSI-high cancers are heterogeneously represented in tumor sub-clones[30], gene rearrangements appear as "founder" events, as they are present in most, if not all, tumor cells. Nevertheless, since defective mismatch repair is also an early event in CRC carcinogenesis, the adenoma-carcinoma sequence should be further elucidated for this rare subtype., Future studies exploring the role of food carcinogens and/or peculiar microbiota components in the right colon are also warranted to clarify the potential link between MSI status and kinase rearrangements.

When compared with negative samples, *ALK, ROS1*, and *NTRK* rearranged tumors show a low frequency of *RAS* and *BRAF* oncogenic mutations. A low prevalence of BRAF V600E mutation was reported in the group of negative tumors (5.8%), probably as a consequence of the poor

prognosis and rapid progression of *BRAF* mutant tumors, preventing these patients to receive later lines of therapy and therefore to be screened for phase 1 trials. Therefore, we were unable to identify a statistically significant difference in terms of *BRAF* mutations between rearranged and not rearranged tumors (p=1.000) in the present series. However, the observation that *ALK*, *ROS1* and *NTRK* rearrangements co-occur rarely with other common driver events in the RTK-RAS pathway, and specifically *RAS* and *BRAF* codon 600 mutations, supports the hypothesis that gene fusions drive oncogene addiction. Indeed, previous reports indicate that *NTRK1* and *ALK* rearranged CRC preclinical models and patients respond to pharmacological blockade of the fusion kinase [6,11,15,19,20]. In spite of the relatively low prevalence of gene fusions, the identification of patients with tumors bearing these alterations may be simplified and enriched by the evaluation of four simple and easy-to-collect variables (i.e. primary tumor location, *RAS*, *BRAF* and MSI-high status), which are available for the vast majority of patients. Therefore, in an evidence-based perspective of resource sparing, the molecular screening for gene rearrangements should not be denied to patients with *RAS* and *BRAF* wild-type and/or MSI-high mCRC.

A high prevalence of RNF43 frameshift mutations was reported among *ALK*, *ROS1* and *NTRK* rearranged tumors, though in the absence of concomitant BRAF V600E mutations, thus suggesting that gene rearrangements may act as driver events alternative to *BRAF* in the tumorigenesis of MSI-high right-sided tumors carrying *RNF43* alterations. Since porcupine inhibitors are being developed to suppress paracrine WNT-driven growth of *RNF43* mutant tumors (https://clinicaltrials.gov/ct2/show/NCT02278133), our findings may provide a rationale for co-targeting tyrosine kinase oncogenic fusions as well as the WNT pathway in this rare tumor subset.

Closely recalling the long "*BRAF* history", we found that gene fusions occurring in mCRCs are associated with unfavorable outcome. However, it must be pointed out that patients with MSI-high mCRCs have worse OS independently from the co-occurrence of *BRAF* V600E mutation [28]. Therefore, given the association of *ALK, ROS1* or *NTRK* rearrangements with MSI-high status and the mutual exclusivity with codon 600 *BRAF* mutations, our findings may partly explain the

aggressive behaviour of MSI-high *BRAF* wild-type mCRCs. The same observations are true for the potential contribution of gene fusion to the poor prognosis of some right-sided mCRCs [31].

Again, consistent with previous findings regarding *BRAF* V600E mutations [32], *ALK*, *ROS* and *NTRK* rearranged tumors seem not to derive benefit from anti-EGFR monoclonal antibodies,, thus confirming preclinical observations [19]. Given the very low frequency of gene fusions in mCRC, the validation of this finding is quite unrealistic. However, these results are supported by a strong biologic rationale and may contribute to explain – at least in part - the limited activity of anti-EGFRs in right-sided, *RAS* and *BRAF* wild-type tumors [33]. From a clinical perspective, it seems therefore reasonable to offer an intensive first-line regimen, such as the triplet FOLFOXIRI plus bevacizumab to patients with right-sided, *ALK, ROS1*, and *NTRK* rearranged mCRCs [34], based on their aggressive behaviour, and in line with current recommendations for *BRAF* V600E mutant tumors.

Our observations argue that the early enrolment of patients with tumors bearing *ALK, ROS1* and *NTRK* rearrangements in clinical trials with matched targeted agents should be highly encouraged, as this subset of patients may in fact be uniquely poised to benefit from targeted strategies. Nevertheless, benefit from targeted strategies against ALK, ROS1, and TrkA-B-C may be transient and mechanisms of acquired resistance may occur early [17,20]. This is quite reasonable particularly when considering the impressive mutational burden of MSI-high tumors that may promote in these tumors the early emergence of acquired resistance.

The combination of targeted agents and immunotherapy approaches in MSI-high rearranged tumors may be a promising strategy to be further investigated, supported by a strong molecular rationale, and by the absence of impact of rearrangements on MSI-high associated immunophenotype.

The major limitation of this study is the choice of the control group. Although a wider series of negative cases, especially those analyzed by MSK-IMPACT or FoundationOne tests, would have been more appropriate, both MSK-IMPACT and FoundationOne are DNA-based assays and do not

completely cover intronic regions, thus making possible to miss some gene fusions. Moreover, clinical data were not available for the vast majority of these patients. Therefore, a cohort of well-annotated patients screened at three Institutions for a phase 1 trial and quite representative of the general population of mCRC patients was adopted as control group.

In conclusion, the features of *ALK*, *ROS1* and *NTRK* rearrangements are somewhat reminiscent of the peculiar traits previously recognized in *BRAF* V600E mutant mCRC. These fusions define a new molecular subtype of mCRC associated with poor prognosis, whose recognition allows a proper tailored management for a new subgroup of patients. The large-scale diffusion of this assessment may be eased by the availability of a multi-step procedure for the detection of gene fusions, starting from a simple IHC test with high sensitivity, or a comprehensive approach able to identify *ALK*, *ROS1*, and *NTRK* rearrangements, as well as other potentially targetable kinase fusions [22]. Finally, while the poor prognosis of rearranged tumors may suggest the adoption of upfront intensive treatments when feasible, new targeted strategies are under investigation and the high prevalence of MSI-high status in rearranged tumors opens the way to evaluate new combination approaches, including targeted (ALK, ROS1, TrkA-B-C) and immunotherapy agents.

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Table 1. Patients' characteristics according to the presence or absence of *ALK, ROS1, NTRK* rearrangements, or specifically for presence or absence of *NTRK* and *ALK* rearrangements.

Characteristics		*ALK, ROS1, NTRK* negative (N=319) N (%)	*ALK, ROS1, NTRK* rearranged (N=27) N (%)	*p**	*NTRK* rearranged (N=13) N (%)	*p*†	*ALK* rearranged (N=11) N (%)	*p*‡
Sex	Female	129 (40.4)	18 (66.7)	0.159	9 (69.2)	**0.047**	7 (63.6)	0.211
	Male	190 (59.6)	9 (33.3)		4 (30.8)		4 (36.4)	
Age	Median	57	64	**0.024**	68	**0.032**	55	0.967
	Range	15-88	40-62		33-73		40-87	
ECOG PS	0	106 (33.4)	9 (64.3)	0.250	2 (25.0)	1.000	3 (75.0)	0.115
	1-2	211 (66.6)	5 (35.7)		6 (75.0)		1 (25.0)	
	NA	2	13		5		7	
Primary tumor location	Right colon	98 (31.0)	20 (80.0)	**<0.001**	10 (90.9)	**<0.001**	8 (72.7)	**0.014**
	Left colon	125 (39.6)	3 (12.0)		0		2 (18.2)	
	Rectum	93 (29.4)	2 (8.0)		1 (9.1)		1 (9.1)	
	NA	3	2		2		0	
Mucinous histology	Yes	40 (12.7)	1 (5.9)	0.706	0	0.602	1 (11.1)	

	No	276 (87.3)	16 (94.1)		8 (100.0)		8 (88.9)	1.000
	NA	3	10		5		2	
Primary tumor resected	Yes	240 (75.2)	19 (86.4)		8 (72.7)		0	
	No	79 (24.8)	3 (13.6)	0.308	3 (27.3)	1.000	8 (100.0)	**<0.001**
	NA	0	5		2		3	
Time to metastases	Synchronous	210 (66.2)	11 (64.7)		5 (62.5)		6 (75.0)	
	Metachronous	107 (33.8)	6 (35.3)	1.000	3 (37.5)	1.000	2 (25.0)	0.723
	NA	2	10		5		3	
Number of metastatic sites	1	161 (50.9)	14 (58.3)		7 (63.6)		6 (54.5)	
	>1	155 (49.1)	10 (41.7)	0.531	4 (36.4)	0.544	5 (45.5)	1.000
	NA	3	3		2		0	
Lung metastases	Yes	129 (40.8)	5 (20.8)		0		4 (36.4)	
	No	187 (59.2)	19 (79.2)	0.053	11 (100.0)	1.000	7 (63.6)	1.000
	NA	3	3		2		0	
Lymph Nodes metastases	Yes	78 (24.7)	11 (45.8)		7 (63.6)		3 (27.3)	
	No	238 (75.3)	13 (54.2)	**0.030**	4 (36.4)	**0.008**	8 (72.7)	0.737
	NA	3	3		2		0	
Liver metastases	Yes	207 (65.5)	10 (41.7)		4 (36.4)		5 (45.5)	
	No	109 (34.5)	14 (58.3)	**0.026**	7 (63.6)	0.058	6 (54.5)	0.204

	NA	3	3		2		0	
Peritoneal metastases	Yes	89 (28.2)	8 (33.3)		5 (45.5)		3 (27.3)	
	No	227 (71.8)	16 (66.7)	0.640	6 (54.5)	0.306	8 (72.7)	1.000
	NA	3	3		2		0	
RAS status	wild-type	155 (51.7)	25 (92.6)		11 (84.6)		9 (81.8)	
	mutated	145 (48.3)	2 (7.4)	**<0.001**	2 (15.4)	**<0.001**	2 (18.2)	0.065
	NA	19	0		0		0	
BRAF status	wild-type	258 (94.2)	26 (96.3)		13 (100.0)		11 (100.0)	
	V600E mutated	16 (5.8)	1 (3.7)	1.000	0	1.000	0	1.000
	NA	45	0		0		0	
MSI status	MSS	148 (91.9)	14 (51.9)		3 (23.1)		4 (36.4)	
	MSI-high	13 (8.1)	13 (48.1)	**<0.001**	10 (76.9)	**<0.001**	7 (63.6)	**<0.001**
	NA	158	0		0		0	

NA: not available. *Comparison of *ALK, ROS1, NTRK* rearranged versus not rearranged tumors; †Comparison of *NTRK* rearranged versus not rearranged tumors; ‡Comparison of *ALK* rearranged versus not rearranged tumors. *ROS1* rearranged tumors were not separately analyzed because of the small sample size (N=3)

Table 2. Association of *ALK, ROS, NTRK* rearrangements and known prognostic baseline characteristics with OS

Characteristics		N	Univariate analyses			Multivariable model		
			HR	95% CI	*p*	HR	95% CI	*p*
ALK, ROS, NTRK status	Negative	316	1	-	-	1	-	-
	Rearranged	20	2.17	1.03-4.57	**<0.001**	2.33	1.10-4.95	**0.020**
Age	-	336	1.04	1.02-1.05	**<0.001**	1.04	1.02-1.07	**<0.001**
ECOG PS	0	112	1	-	-	-	-	-
	1-2	216	1.01	0.72-1.42	0.950	-	-	-
Primary tumor site	Left colon/Rectum	221	1	-	-	1	-	-
	Right colon	113	1.41	1.01-2.00	**0.038**	1.11	0.62-1.98	0.733
Mucinous histology	No	290	1	-	-	-	-	-
	Yes	41	0.97	0.59-1.58	0.885	-	-	-
Primary resection	Yes	257	1	-	-	1	-	-
	No	82	1.51	1.01-2.29	**0.024**	1.69	0.94-3.05	0.079
Time to metastases	Metachronous	113	1	-	**-**	-	-	**-**
	Synchronous	220	1.24	0.88-1.74	0.242	-	-	-
Number of metastatic sites	1	171	1	-	-	-	-	-
	>1	164	1.28	0.93-1.77	0.134	-	-	-
RAS status	Wild-type	173	1	-	-	-	-	-
	Mutated	147	1.31	0.94-1.82	0.117	-	-	-
BRAF status	Wild-type	275	1	-	-	1	-	-
	Mutated	17	2.20	0.97-4.95	**0.058**	0.91	0.35-2.38	0.855
MSI status	MSS	156	1	-	-	1	-	-
	MSI-high	22	2.28	1.09-4.76	**0.005**	1.42	0.63-3.21	0.397

Figure Legends

Figure 1. Study flow-chart.

Top: A total of 27 metastatic colorectal cancer (mCRC) cases with *ALK* (n=11), *ROS1* (n=3) and *NTRK* (n=13) translocations were collected. Patients were retrieved by: Ignyta's phase 1 screening program in Italy, Belgium and South Korea; MAX trial's post-hoc analysis conducted in Australia; Foundation Medicine Inc. (FMI) dataset in USA; Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) screening program in USA. **Bottom left:** Clinicopathological characteristics, *RAS* and *BRAF* status, Mismatch-repair (MMR) status, survival and treatment outcome data in the *ALK, ROS1, NTRK* rearranged population (n=27) were compared with those from a cohort of *ALK, ROS1, NTRK* negative mCRC patients (n=319) included in Ignyta's phase 1 screening program. **Bottom right**: Annotated genetic variants were retrieved from targeted next-generation sequencing analyses of tumor samples (N=27) from *ALK, ROS1, NTRK* rearranged mCRC patients. The number of samples analyzed by different gene panels is shown. Analysis of publicly available RNA sequencing data from the TCGA COADREAD (colorectal) study allowed the identification of 7 additional tumors carrying *ALK* or *NTRK3* translocations. Molecular annotations from TCGA translocated tumors were pooled with those from mCRC patients to increase power of detecting genetic alterations co-existing with *ALK, ROS1, NTRK* rearrangements.

Figure 2. Molecular profile of *ALK, ROS1, NTRK* rearranged colorectal cancer. A. OncoPrint map depicting alterations in top mutated colorectal cancer genes in *ALK, ROS1, NTRK* rearranged cancers (27 cases from this study and 7 samples from TCGA[23]). Individual sample cases are designated by columns (top) and grouped by MMR status, while individual genes are presented by rows. **B.** Gene mutation profiles, excluding silent mutations, were compared between *ALK, ROS1, NTRK* rearranged cancers (27 cases from this study and 7 samples from TCGA) and data previously reported in a large-scale sequencing study of unselected CRC[25]. Grey bars indicate the number of samples that were not sequenced for the indicated genes. **C**. Expression (RNA sequencing data) of selected genes implicated in immunoevasion (gene list was obtained from[25]) in *ALK* or *NTRK3* rearranged tumors identified in TCGA, grouped based on their MMR status. The average expression of non-rearranged TCGA MSI-high CRC samples (n=92) from TCGA is also shown.

Figure 3. Survival in metastatic colorectal cancer patients carrying *ALK, ROS1, NTRK* rearranged tumors. Panel A: Kaplan-Meier curves for overall survival (OS) in patients with *ALK, ROS1, NTRK* rearrangements (n=20; red line) as compared to those with *ALK, ROS1, NTRK* negative tumors (n=316; blue line). **Panel B:** Kaplan-Meier curves for overall survival (OS) in patients with *ALK, ROS1, NTRK* rearrangements and MMR proficient status (n=11; red line) or patients with *ALK, ROS1, NTRK* rearrangements and MMR deficient status (n=9; green line) as compared to those with *ALK, ROS1, NTRK* negative tumors (n=316; blue line).

Contributors

Study design: FP, FDN, CC

Data collection and patients' recruitment: FP, FDN, GF, CA, STK, DM, JS, BM, PJS, MC, LL, VAM, RS, RB, FM, AA, CC

Data analysis and interpretation: FP, FDN, ABS, JL, ST, ASB, JH, JC, LN, NT, MM, JSR, SS, AB, SMA, AF, FDB, CC

Manuscript writing: all authors

Manuscript revision and approval: all authors

Declaration of interests

F.P. is a consultant/advisory board member for Roche, Amgen, Eli-Lilly, Bayer, Sanofi. S.S. is an advisory board member for Amgen, Roche, Novartis, Eli-Lilly, Bayer, Sanofi, Merck, Merrimack. A.B. is a member of advisory boards for Horizon Discovery and Trovagene. A.F. is a consultant/advisory board member for Bayer, Roche, Amgen, Eli-Lilly, Merck Serono, Sanofi, Servier. F.d.B. is a consultant/advisory board member for Roche, Amgen, Novartis, Celgene, Boehringer-Ingelheim. C.C. is a consultant/advisory board member for Roche, Amgen, Eli-Lilly, Bayer, Merck Serono.

A.B.S., J.S., P.J.S., V.A.M., J.S.R., and S.M.A. are employees and have equity interest in Foundation Medicine, Inc. J.C., D.M., B.M., M.C., R.S. are employees and have equity interest in Ignyta, Inc

All other authors declare no potential competing interests

Figure 1

A

	***NTRK* fusions N=13**	***ALK* fusions N=11**	***ROS1* fusions N=3**
Ignyta's STARTRK-1 phase 1 study ***screening program*** †	*LMNA-NTRK1* (n=1) [7] *TPM3-NTRK1* (n=3*) [8] *SCYL3-NTRK1* (n=1*)	*CAD-ALK* (n=1) [9] *EML4-ALK* (n=2*) [6]	
MAX trial ***post-hoc analysis*** ‡		*EML4-ALK* (n=1) [4]	*Unknown-ROS1* (n=1) [4] *SLC34A2-ROS1* (n=1) [4]
Foundation Medicine Inc, ***Clinical database***	*TPM3-NTRK1* (n=4*) *ETV6-NTRK3* (n=2) [12] *LMNA-NTRK1* (n=1*)	*CAD-ALK* (n=2*) [6] *EML4-ALK* (n=1) [11] *CENPF-ALK* (n=1) [11] *PRKAR1B-ALK* (n=1) [11] *MAPRE3-ALK* (n=1) [11] *STRN-ALK* (n=1) [11]	*GOPC-ROS1* (n=1*)
MSK-IMPACT ***screening program***	*LMNA-NTRK1* (n=1*)		

***ALK, ROS, NTRK* rearranged mCRCs N=27**

B

Clinicopathological characteristics available, *N=27*
RAS, BRAF status available, *N=27*
MMR status available, *N=26*
Survival data available, *N=20*
Treatment data available, *N=14*

***ALK, ROS, NTRK* negative mCRCs N= 319**

C

NGS data available N=27, by means of:
- Minerva panel, N= 11
- Foundation Medicine Inc. (FMI) panel, N= 15
- MSK-IMPACT, N=1

+

ALK, NTRK rearranged CRC samples in TCGA N= 7

Figure 2

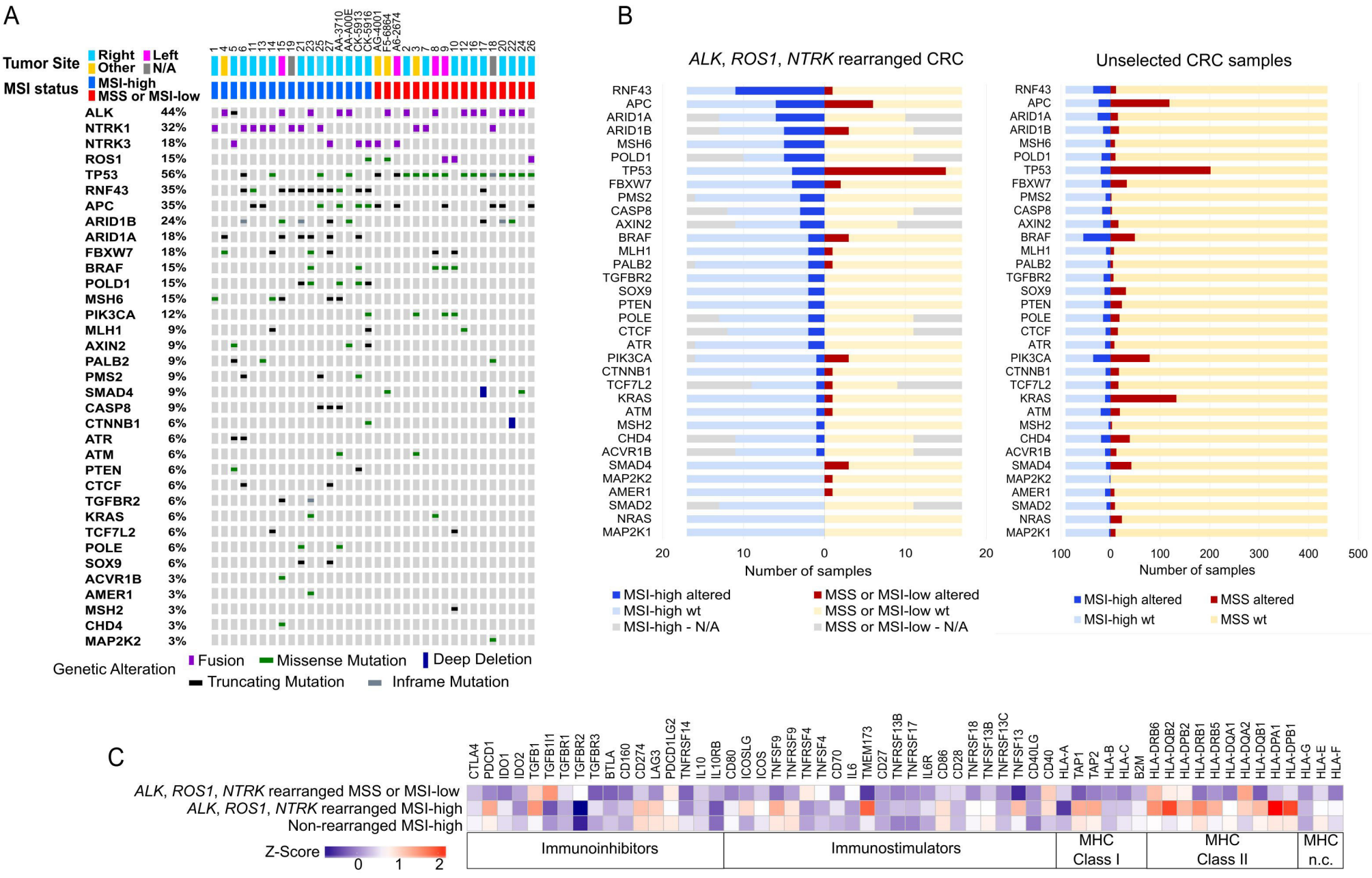
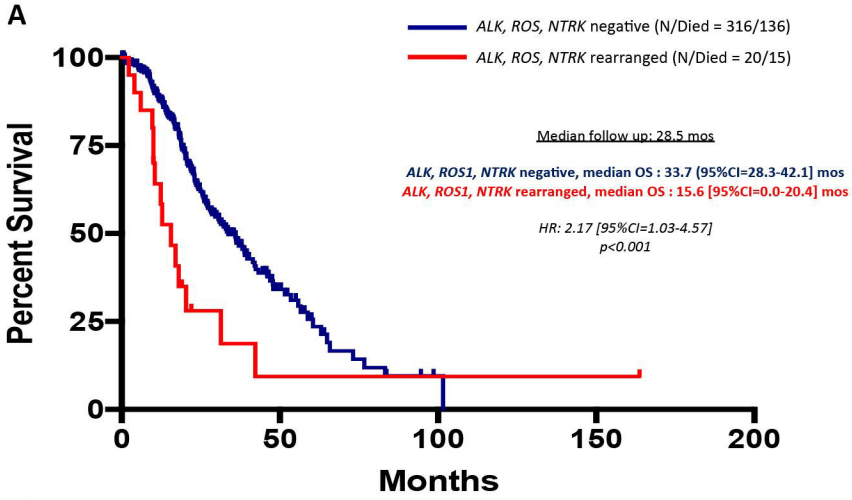

A
Tumor Site
Right
Left
Other
N/A
MSI status
MSI-high
MSS or MSI-low
ALK 44%
NTRK1 32%
NTRK3 18%
ROS1 15%
TP53 56%
RNF43 35%
APC 35%
ARID1B 24%
ARID1A 18%
FBXW7 18%
BRAF 15%
POLD1 15%
MSH6 15%
PIK3CA 12%
MLH1 9%
AXIN2 9%
PALB2 9%
PMS2 9%
SMAD4 9%
CASP8 9%
CTNNB1 6%
ATR 6%
ATM 6%
PTEN 6%
CTCF 6%
TGFBR2 6%
KRAS 6%
TCF7L2 6%
POLE 6%
SOX9 6%
ACVR1B 3%
AMER1 3%
MSH2 3%
CHD4 3%
MAP2K2 3%
Genetic Alteration
Fusion
Missense Mutation
Deep Deletion
Truncating Mutation
Inframe Mutation
B
ALK, ROS1, NTRK rearranged CRC
Unselected CRC samples
Number of samples
MSI-high altered
MSS or MSI-low altered
MSI-high wt
MSS or MSI-low wt
MSI-high - N/A
MSS or MSI-low - N/A
MSS altered
MSS wt
C
ALK, ROS1, NTRK rearranged MSS or MSI-low
ALK, ROS1, NTRK rearranged MSI-high
Non-rearranged MSI-high
Z-Score
Immunoinhibitors
Immunostimulators
MHC Class I
MHC Class II
MHC n.c.

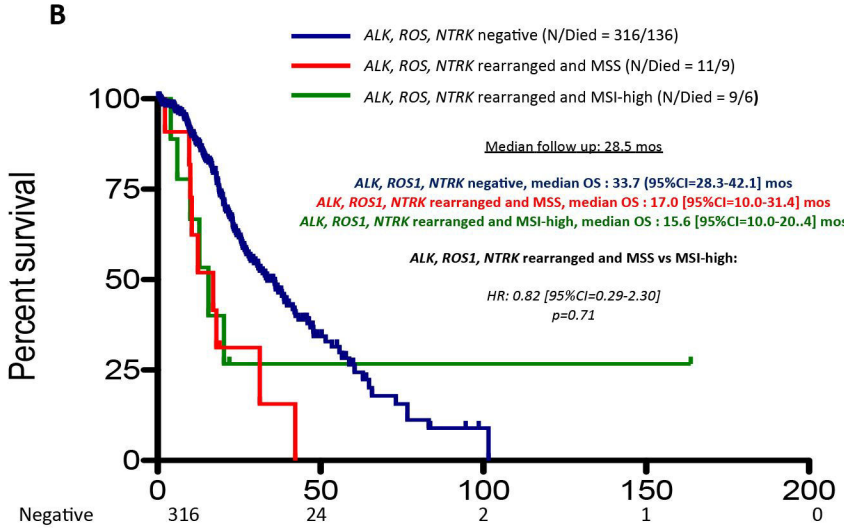
Figure 3

A



Negative	316	24	2	1	0
Rearranged	20	2	1	1	0

B



Negative	316	24	2	1	0
Rearr. MSS	11	0	0	0	0
Rearr. MSI-high	9	2	1	1	0

Supplementary Material

Supplement to: Pietrantonio F, Di Nicolantonio F, Schrock et al. ***ALK*, *ROS1* and *NTRK* rearrangements define a new subtype of metastatic colorectal cancer**

Contents

Supplementary Methods

Patients screening and translational analyses

1 - STARTRK1 phase 1 study by Ignyta®. In the first step, all screened samples were submitted to Immunohistochemistry (IHC) staining, as previously described [1]. Briefly, a cocktail of antibodies targeted to the C-terminal domains of pan-Trk (including TrkA, TrkB, TrkC, Cell Signaling, clone C17F1, 1:25 dilution), ROS1 (Cell Signaling, clone D4D6, 1:500 dilution) and ALK (Cell Signaling, clone D5F3, 1:500 dilution) was used with a single diaminobenzidine (DAB) reporter system. The presence of staining indicates the elevation of expression for at least one of the proteins targeted by the antibody cocktail. In the second step, all samples scored positive for IHC staining were tested by RNA-based next generation sequencing to determine the presence/absence of a gene fusion. The presence and nature of gene rearrangements/fusions was determined by RNA sequencing using a method previously described [1]. Total nucleic acid (a combination of both DNA and RNA) was isolated from FFPE tissues (Agencourt, Beckman) and sequencing libraries were generated using an anchored multiplex PCR (AMP) method [1,2]. RNA quality and amplifiable ability of the extracted material was assessed as previously described [1]. Briefly, the PreSeq RNA QC assay, which uses qPCR analysis of the housekeeping gene, VCP was performed on all samples. The RNA quality assessment was used to determine the potential for a false negative result from specimens where the RNA was fragmented to a degree that a gene rearrangement could not be amplified or mapped reliably. Sequencing was performed on the Illumina MiSeqDx platform (Illumina, San Diego, CA).

For patients screened at Ignyta, DNA next generation sequencing (NGS) analysis was performed through the custom "Minerva" panel, which interrogated 263 genes resumed in the list below:

ABL1	BRIP1	CHEK2	ERG	GLI1	IHH	MET	NTF4	RUNX1	TSC2
AKT1	BTC	CHMP2A	ESR1	GNA11	IKZF1	MIB1	NTRK1	RUNX1T1	TYRO3
AKT2	BTK	CREBBP	EZH2	GNAQ	IL12A	MKI67	NTRK2	SDHB	VCAM1
AKT3	CAD	CRKL	FANCA	GPI	IL12B	MLH1	NTRK3	SETD2	VCP
ALDH1A1	CBFB	CSF1R	FANCB	GSK3B	IRF1	MPL	PALB2	SHH	VEGFA
ALK	CCL2	CSF3R	FANCC	GTSE1	IRS2	MS4A1	PDCD1	SMAD4	VHL
AMER1	CCL5	CTLA4	FANCD2	GZMA	JAG1	MSH2	PDGFRA	SMO	VIM
APC	CCND1	CTNNB1	FANCF	GZMB	JAK1	MSH6	PDGFRB	SNAI1	WNT1
AR	CCND2	CX3CL1	FANCG	GZMH	JAK2	MTOR	PIK3CA	SOX2	WNT10A
ATAD2	CCND3	CXCL10	FANCI	HBEGF	KDR	MYC	PIK3CG	SOX9	WNT10B
ATM	CD22	CXCL11	FANCL	HDAC1	KEAP1	MYCN	PIK3R1	SRC	WNT2B
ATR	CD274	CXCL9	FANCM	HDAC4	KIT	MYD88	PMS2	STAT3	WNT3
AURKA	CD3D	CXCR3	FAS	HES1	KMT2A	NANOG	PRKCG	STAT6	WNT4
AXIN1	CD4	CXCR4	FBXW7	HGF	KRAS	NF1	PRKCI	STK11	WNT5A
AXIN2	CD47	DBF4	FGF23	HNF1A	LAG3	NF2	PTCH1	SYK	WNT7A
AXL	CD68	DDR2	FGFR1	HOXA9	LNX2	NFE2L2	PTEN	TBX21	WNT8A

BAP1	CD79B	DOT1L	FGFR2	HRAS	LYN	NFKBIA	RAB7A	TCF3	WNT9A
BARD1	CD8A	EGF	FGFR3	ICAM1	MAP2K1	NGF	RAD50	TCF4	WNT9B
BCL2	CDC7	EGFR	FGFR4	IDH1	MAP2K2	NGFR	RAD51	TCF7L2	WT1
BCL6	CDH1	EP300	FH	IDH2	MAP2K4	NKX2-1	RAF1	TGFA	XPO1
BDNF	CDH2	EPGN	FLT1	IDO1	MAPK1	NOTCH1	RALA	TGFBR2	
BRAF	CDK4	EPHA2	FLT3	IFNG	MAPK3	NOTCH2	RALB	TNFRSF4	
BRCA1	CDK6	EPHA3	FLT4	IGF1	MCL1	NOTCH3	RARA	TNIK	
BRCA2	CDKN1B	ERBB2	FOXP3	IGF1R	MCM2	NPM1	RB1	TOP1	
BRD3	CDKN2A	ERBB3	GAS6	IGF2	MDM2	NRAS	RET	TOP2A	
BRD4	CEBPA	ERBB4	GATA3	IGF2R	MEN1	NRG1	RNF43	TP53	
BRDT	CHEK1	EREG	GATA6	IGFBP1	MERTK	NTF3	ROS1	TSC1	

2 - Retrospective translational study of the Australian MAX trial, as previously described [3].

3 - Samples tested by Foundation Medicine were assayed with a validated comprehensive genomic profiling (CGP) platform during the course of clinical care at the request of the treating physician. DNA was extracted from 40 microns of FFPE sections, and CGP was performed on hybridization-captured, adaptor ligation based libraries to a mean coverage depth of >650X for 236 or 315 cancer-related genes plus select introns from 19 or 28 genes frequently rearranged in cancer as described previously [4]. All classes of genomic alterations (GA) were identified including base pair substitutions, insertions/deletions, copy number alterations, and rearrangements. Microsatellite instable (MSI-H) or stable (MSS) status as a measure of mismatch repair deficiency was determined using a proprietary computational algorithm. Tumors were classified as microsatellite instable (MSI-H) or microsatellite stable (MSS) using a principal component 1 cutoff value of less than -8.5 or greater than -4, respectively [5].

4 - Samples tested by Memorial Sloan Kettering Cancer Center underwent analysis by the clinically validated MSK-IMPACT assay. This hybridization-based next generation sequencing assay interrogates all exons and select introns and promoters of over 340 cancer-related genes. Tumor samples are sequenced against matched normal samples and only somatic alterations including structural variants, mutations, and copy number alterations are reported. Further details about this assay have been published by Cheng et al. [6].

In silico analysis of the TCGA data (ALK, ROS1, NTRK fusion search in TCGA-COAD-READ)

FPKM-normalized transcriptomic profiles were downloaded from the Genomic Data Commons Data Portal (https://gdc-portal.nci.nih.gov) for the tumor samples in the TCGA-COAD and TCGA-READ datasets and the z-score for each gene was calculated. Tumors in the 95th percentile for *ALK*, *NTRK1*, *NTRK2*, *NTRK3* or *ROS1* gene expression were selected for further analyses, since outlier

kinase expression is often driven by fusion transcripts [7,8]. For the 154 tumor samples carrying outlier expression in one of the selected kinases (as shown in Supplementary Fig. 2 and listed in Supplementary Table 4), RNAseq reads were downloaded from the Genomic Data Commons Data Portal. Reads were aligned using the BWA-mem [9] algorithm to hg19 human reference genome, then all the non-perfect alignments falling on the genes of interest were selected and aligned using BLAT [10] with tileSize=11 and stepSize=5. The resulting alignment was post-processed to detect chimeric alignments, by applying the following criteria: i) each fusion partner must have at least 15 nucleotides mapped of the respective end of the read; ii) the two parts of the read must map to different genes; iii) at least one of the two fusion breakpoints must be on the exon boundary. Due to the short read length (ranging from 48 to 76, Supplementary Table 4), it was not possible to impose a threshold on the number of reads supporting each fusion breakpoint. After the first gene-specific analysis, we did a cross-validation on the entire transcriptome using FusionMap[11]. In addition to the six fusion transcripts found on selected genes using our custom-built pipeline, FusionMap was able to identify also a previously reported VPS18-NTRK3 translocation[12].

Characterization of the novel *SCYL3-NTRK1* fusion

For Patient #13 harboring the novel fusion, *SCYL3-NTRK1*, a set of PCR primers was generated to further confirm the result (SCYL3: 5'- GGAGGAGAACGAACCAAGAT; NTRK1: 5'- CATGAAATGCAGGGACATGG). Total nucleic acid was reverse-transcribed and amplified by PCR using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (ThermoFisher, Carlsbad, CA). The PCR products were assessed on a 2100 Bioanalyzer electrophoresis system (Agilent, Santa Clara, CA). A parallel no template control was also included to determine the presence of any background hybridization.

Criteria for evaluation of primary resistance to anti-EGFR monoclonal antibodies

To assess the association of *ALK*, *ROS1* and *NTRK* status with primary resistance to anti-EGFR MoAbs, we restricted the analysis to *RAS* and *BRAF* wild-type patients receiving cetuximab or panitumumab as single agents or in combination with irinotecan, only in strictly defined irinotecan-refractory patients (i.e. those with documented disease progression during or within three months from the last irinotecan-containing therapy). We excluded patients receiving an anti-EGFR agent in combination with chemotherapy, except in the case of disease progression as best response indicating primary resistance to the whole treatment. Thus, we were able to focus on the true impact of *ALK, ROS1* and *NTRK* translocations on treatment resistance.

Supplementary Table S1. *ALK, ROS1, NTRK* rearranged cases described in the literature.

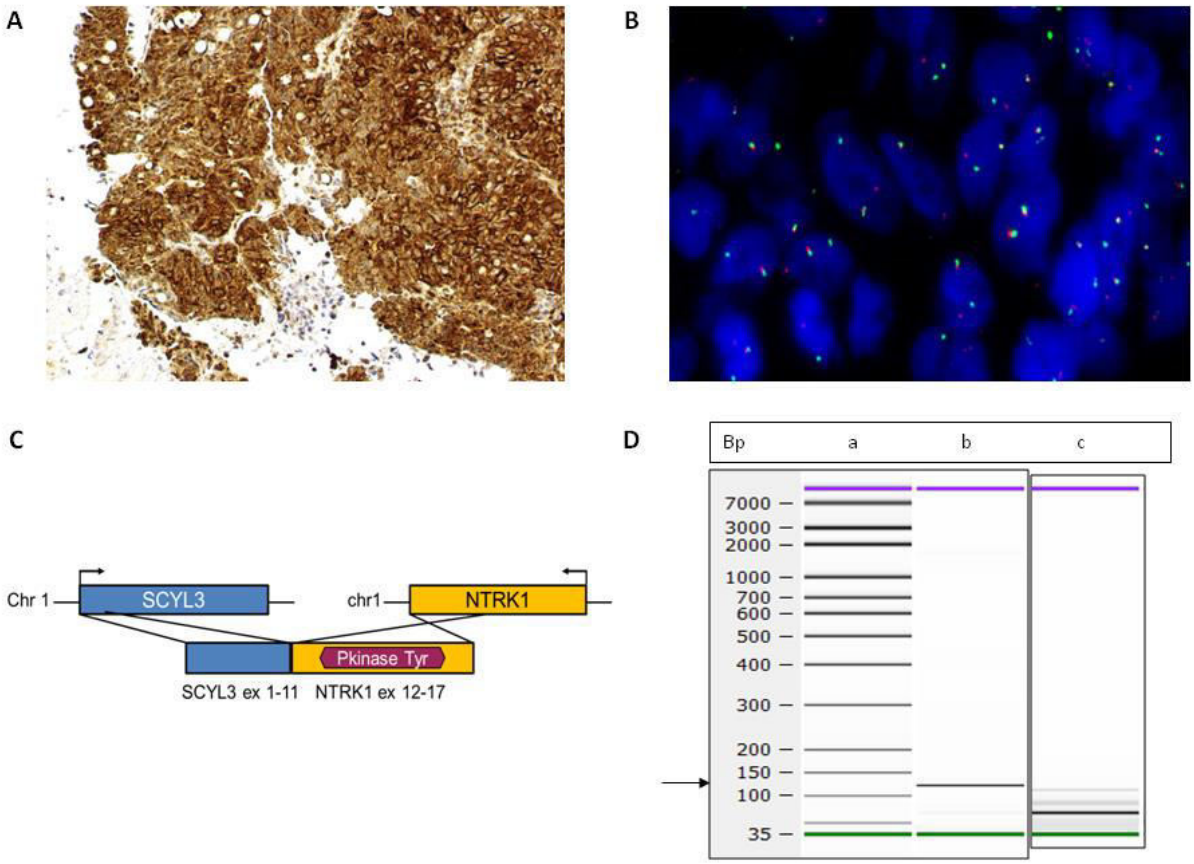
Reference	***NTRK* fusions N=9**	***ALK* fusions N=13**	***ROS1* fusions N=2**	**Retrieved case**
Lin et al, 2009 [2]		*EML4-ALK* *EML4-ALK*		No No
Lipson et al, 2012 [3]		*C2orf44-ALK*		No
Aisner et al, 2014 [4]		*EML4-ALK*	 *SLC34A2-ROS1* *Unknown-ROS1*	Yes Yes Yes
Houang et al, 2015 [5]		*PPP1R21–ALK*		No, stage II
Créancier et al, 2015 [6]	*TPR-NTRK1* *TPM3-NTRK1*			No, stage II No, stage II
Lee J et al, 2015 [6]		*CAD-ALK* *EML4-ALK*		Yes Yes
Sartore Bianchi et al, 2015 [7]	*LMNA-NTRK1*			Yes
Lee S et al, 2015 [8]	*TPM3-NTRK1* *TPM3-NTRK1*			Yes Yes
Amatu et al. 2015 [9]		*CAD-ALK*		Yes
Park et al. 2016 [10]	*LMNA-NTRK1* *TPM3-NTRK1* *TPM3-NTRK1*			No, stage II No, stage III No
Yakirevich et al. 2016 [11]		*STRN-ALK* *CENPF-ALK* *MAPRE3-ALK* *EML4-ALK* *PRKAR1B-ALK*		Yes Yes Yes Yes Yes
Hechtman et al. 2016 [12]	*ETV6-NTRK3*			Yes

Supplementary Table S2. List of patients bearing *ALK, ROS1, NTRK* rearrangements with retrieving source, clinical center, identified gene fusion and NGS panel used.

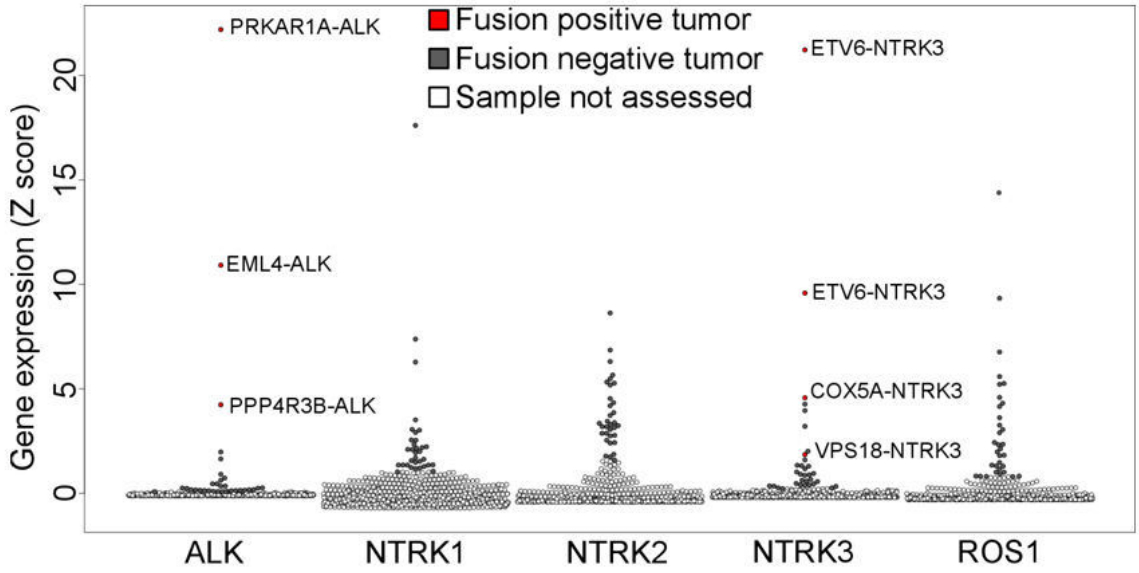
Patient	Retrieving source	Clinical center	Gene Fusion	NGS panel
#1	Ignyta Inc.	SMC, South Korea	*TPM3-NTRK1*	Minerva panel
#2	Ignyta Inc.	SMC, South Korea	*EML4-ALK*	Minerva panel
#3	Ignyta Inc.	SMC, South Korea	*TPM3-NTRK1*	Minerva panel
#4	Foundation Medicine, MA, USA	SMC, South Korea	*CAD-ALK*	FMI panel
#5	Foundation Medicine, MA, USA	MSKCC, NYC, USA	*ETV6-NTRK3*	FMI panel
#6	MSKCC, NYC, USA	MSKCC, NYC, USA	*LMNA-NTRK1*	MSK-IMPACT
#7	Foundation Medicine, MA, USA	MSKCC, NYC, USA	*LMNA-NTRK1*	FMI panel
#8	Austin Health, Australia	MAX study Investigators	*C2orf44-ALK*	Minerva panel
#9	Austin Health, Australia	MAX study Investigators	*Unknown-ROS1*	Minerva panel
#10	Austin Health, Australia	MAX study Investigators	*SLC34A2-ROS1*	Minerva panel
#11	Ignyta Inc.	NCC, Italy	*LMNA-NTRK1*	Minerva panel
#12	Ignyta Inc.	NCC, Italy	*CAD-ALK*	Minerva panel
#13	Ignyta Inc.	INT, Italy	*SCYL3-NTRK1*	Minerva panel
#14	Ignyta Inc.	INT, Italy	*TPM3-NTRK1*	Minerva panel
#15	Ignyta Inc.	UHG, Belgium	*EML4-ALK*	Minerva panel
#16	Foundation Medicine, MA, USA	Unknown	*CENPF-ALK*	FMI panel
#17	Foundation Medicine, MA, USA	Unknown	*PRKAR1B-ALK*	FMI panel
#18	Foundation Medicine, MA, USA	Unknown	*TPM3-NTRK1*	FMI panel
#19	Foundation Medicine, MA, USA	Unknown	*TPM3-NTRK1*	FMI panel
#20	Foundation Medicine, MA, USA	Unknown	*EML4-ALK*	FMI panel
#21	Foundation Medicine, MA, USA	Unknown	*TPM3-NTRK1*	FMI panel

#22	Foundation Medicine, MA, USA	Unknown	*MAPRE3-ALK*	FMI panel
#23	Foundation Medicine, MA, USA	Unknown	*STRN-ALK*	FMI panel
#24	Foundation Medicine, MA, USA	Unknown	*CAD-ALK*	FMI panel
#25	Foundation Medicine, MA, USA	Unknown	*TPM3-NTRK1*	FMI panel
#26	Foundation Medicine, MA, USA	Unknown	*GOPC-ROS1*	FMI panel
#27	Foundation Medicine, MA, USA	Unknown	*ETV6-NTRK3*	FMI panel

Supplementary Figure 1. Identification and characterization of a novel *SCYL3-NTRK1* in a CRC sample. Panel A: Immunohistochemistry (IHC) staining of tissue from Patient #13 using a cocktail of pan-Trk, ROS1 and ALK antibodies and a single DAB detection system. Strong staining intensity was seen in almost 100% of tumor nuclei indicating the elevated expression of at least one of the targeted proteins. **Panel B**: *NTRK1* FISH (Abnova SPEC NTRK1) was performed on the same specimen and resulted in break-apart positivity for the *NTRK1* gene in 100% of nuclei. **Panel C**: An RNA-based NGS assay using AMP-technology was performed to identify the fusion/fusion partner. This patient exhibited an intrachromosomal inversion and rearrangement that leads to a novel in-frame fusion of *SCYL3* exon 11 to exon 12 of *NTRK1* (upstream of the NTRK1 kinase domain in exons 13-17). **Panel D**: To confirm the novel fusion, RT-PCR was performed using primers specific to the *SCYL3-NTRK1* gene rearrangement. Lane a is a nucleic acid size ladder, annotated in base pair sizes by the column titled 'Bp'; lane b is the RT-PCR product obtained from the patient specimen using rearrangement primers. The arrow indicates the specific RT-PCR product, which migrated at the expected 126 bp size; lane c is a no template control using the same primers as in lane b, which resulted in absence of a PCR product at the expected 126 bp (the strongest product generated migrates at 66 bp).

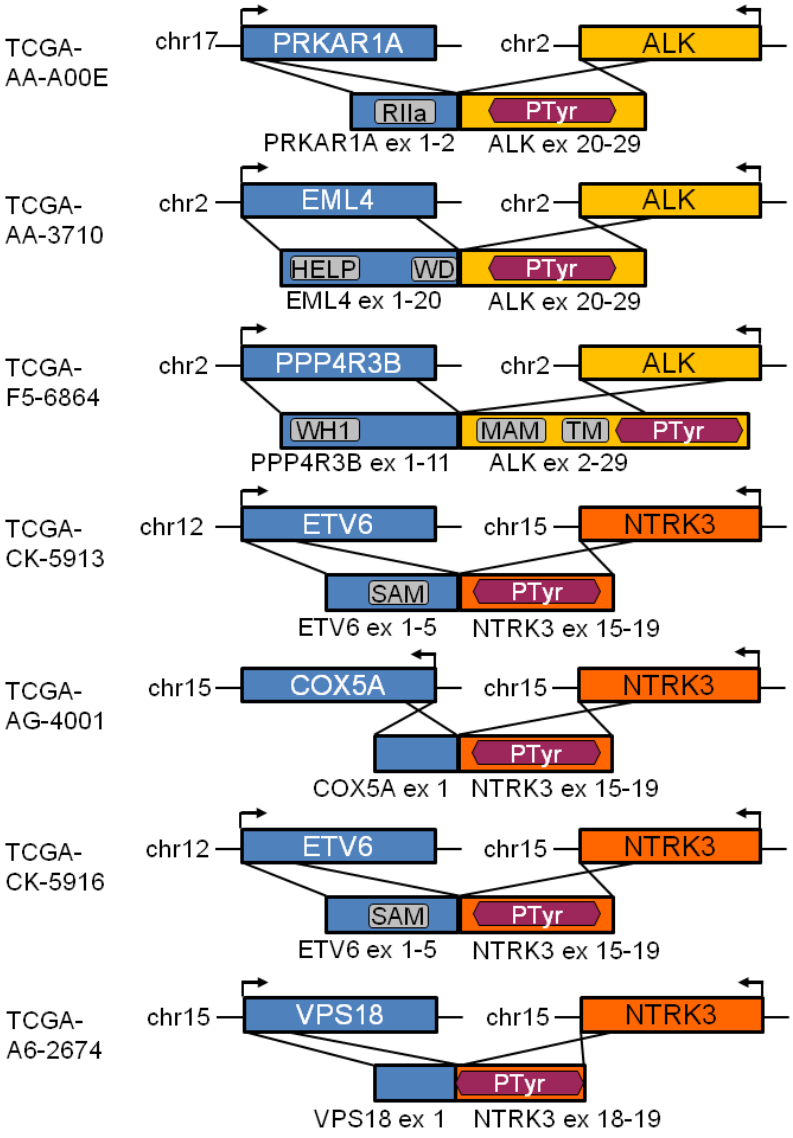


Supplementary Figure 2. Identification of gene fusions in TCGA colorectal cancer samples by outlier kinase analysis. Scatter-plot representation of transcriptional outlier kinases in TCGA CRC samples (N = 644). Grey coloured circles indicate 154 samples (listed in Supplemental Table S4) carrying outlier *ALK, NTRK1, NTRK2, NTRK3, ROS1* gene expression, defined as the 95th percentile for each gene based on z-score normalization. Gene fusion identification in the RNA sequencing reads from these 154 samples was performed by applying a custom pipeline (see Online Methods) and the FusionMap[1] algorithm. A total of 7 fusions (red circles) in the selected kinases were found.

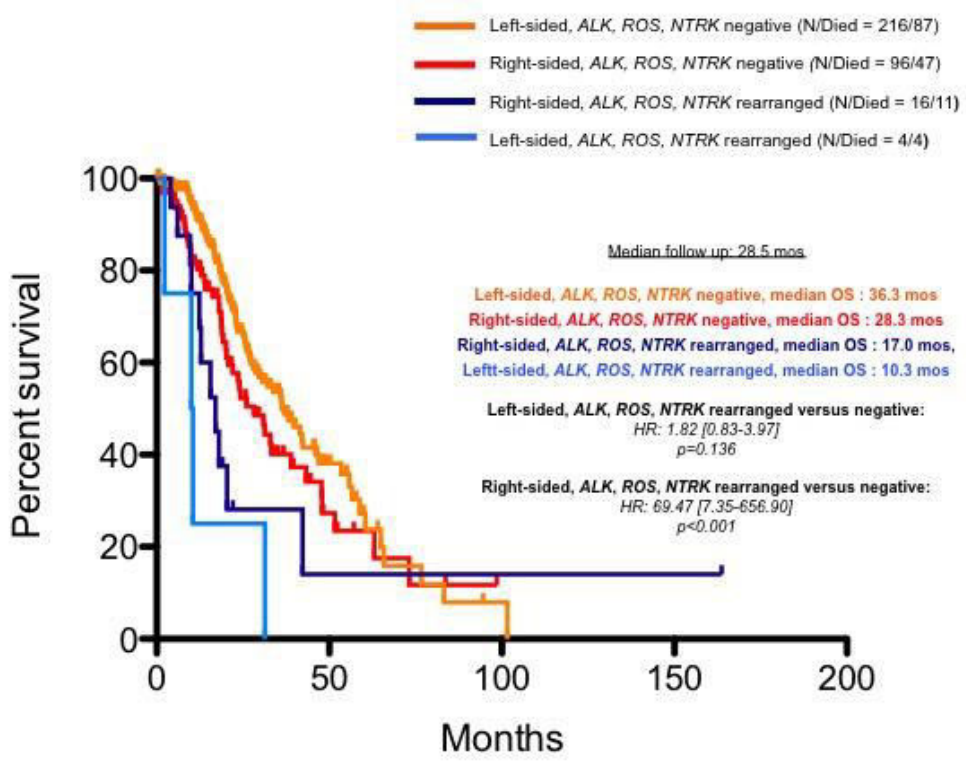


1. Ge, H., *et al.* FusionMap: detecting fusion genes from next-generation sequencing data at base-pair resolution. *Bioinformatics* 27, 1922-1928 (2011).

Supplementary Figure 3. Putative fusion diagrams for each TCGA CRC sample based on publicly available RNA sequencing data. All fusions include the ALK or NTRK3 full tyrosine kinase domain (shown in purple), with the exception of sample TCGA-A6-2674, in which only a portion of the kinase domain was retained. Genomic partner (depicted in blue) is on the left and tyrosine kinase receptor is on the right. Arrows indicate the direction of transcription for each gene. Chromosomes and exons (ex) are also indicated. 1, PRKAR1A–ALK fusion containing a portion of the PRKAR1A regulatory subunit of type II PKA R-subunit (RIIa). 2, EML4–ALK fusion containing the EML4 Hydrophobic EMAP-like protein (HELP) motif and a portion of the WD40 domain. 3, PPP4R3B–ALK fusion containing also the meprin/A5/mu (MAM) and the transmembrane (TM) domains of ALK. 4, ETV6–NTRK3 fusion. 5, COX5A-NTRK3 fusion. 6, ETV6–NTRK3 fusion. 7, VPS18-NTRK3 fusion, in which only a portion of the kinase domain of NTRK3 is retained. Other abbreviations are as follows: PP4R3B, protein phosphatase 4 regulatory subunit 3B; SAM, Sterile alpha motif (SAM)/Pointed domain; HELP, Hydrophobic EMAP-Like Protein motif WD, WD40 repeat (also known as the beta-transducin repeat); WH1, WASp Homology domain 1; MAM, meprin/A5/mu domain; TM, transmembrane domain.

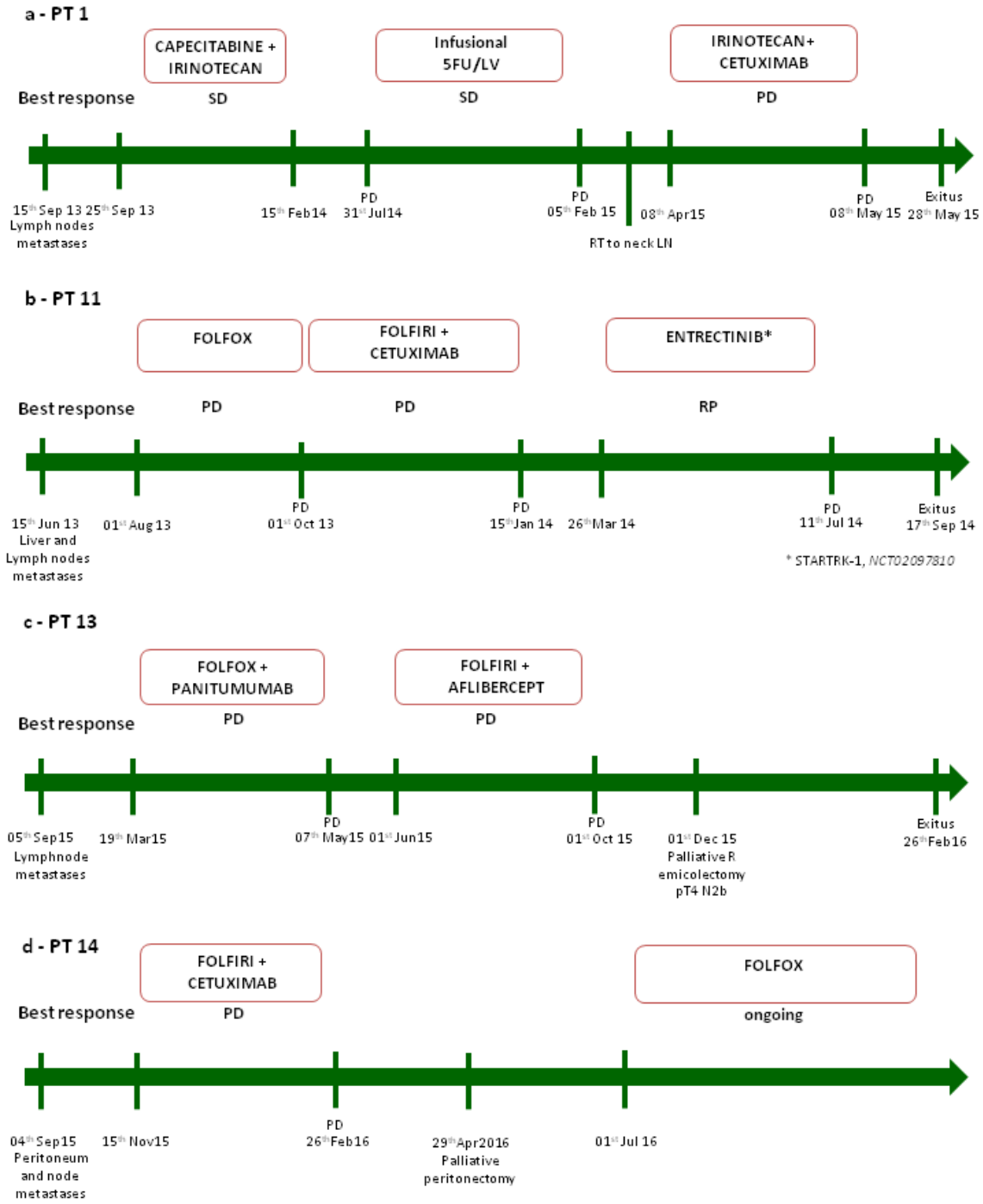


Supplementary Figure 4. Survival in metastatic colorectal cancer patients in subgroups defind by *ALK, ROS1, NTRK* rearrangements and primary tumor location. Kaplan-Meier curves for overall survival (OS) in patients with left-sided primary and *ALK, ROS1, NTRK* rearranged tumors (n=4; light blue line) as compared to those with left-sided primary and *ALK, ROS1, NTRK* negative tumors (n=216; orange line) and in patients with right-sided primary and *ALK, ROS1, NTRK* rearranged tumors (n=16; blue line) as compared to those with right-sided primary and *ALK, ROS1, NTRK* negative tumors (n=96; red line).

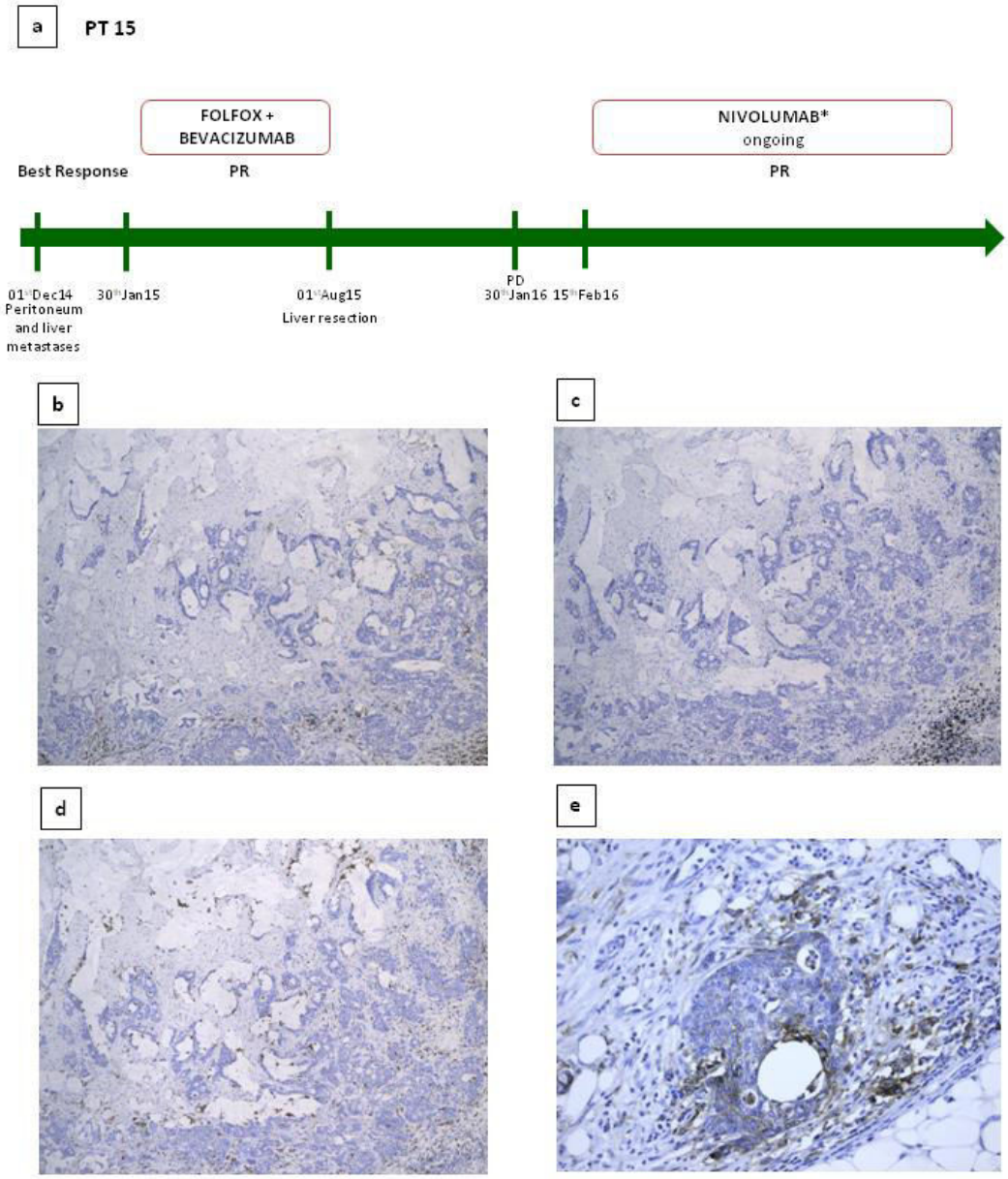


Left - *ALK, ROS, NTRK* neg.	216	17	1	0	0
Right - *ALK, ROS, NTRK* neg.	96	8	1	0	0
Right - *ALK, ROS, NTRK* rearr.	16	2	2	1	0
Left - *ALK, ROS, NTRK* rearr.	4	0	0	0	0

Supplementary Figure 5. Summary of the clinical history of the four patients (N°1, 11, 13 and 14) evaluable for response to anti-EGFR monoclonal antibodies (cetuximab or panitumumab). Shaded boxes indicate periods of administration of the indicated chemotherapeutic agents. Blue vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsy, as well as dates of tumor assessment by radiological imaging. As shown, all evaluable patients had progressive disease to anti-EGFR-based therapy.



Supplementary Figure 6. Summary of the clinical history and immunohistochemical study of the patient (N°15) responding to immune checkpoint inhibition. Panel A. Summary of the clinical history of the patient with EML4-ALK fusion and MMR deficient status (N°15) receving anti-PD-1 immunotherapy with nivolumab. Shaded boxes indicate periods of administration of the indicated chemotherapeutic agents. Blue vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsy, as well as dates of tumor assessment by radiological imaging. As shown, the patients had partial response to anti-PD-1 which is still ongoing. **Panel B.** Positive immunohistochemical staining for CD4. **Panel C.** Positive immunohistochemical staining for CD8. **Panel D.** Positive immunohistochemical staining for CD68. **Panel E.** Positive immunohistochemical staining for PD-L1 in >50% of tumor cells.



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