

# The Potential Role of Genomic Signature in Stage II Relapsed Colorectal Cancer (CRC) Patients: A Mono-Institutional Study

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**Purpose:** The absolute benefit of adjuvant chemotherapy in stage II CRC is only 3–4%. The identification of biomarkers through molecular profiling could identify patients who will more benefit from adjuvant chemotherapy.

**Patients and Methods:** This retrospective analysis examined tissue blocks from 17 patients affected by relapsed stage II CRC, whose comprehensive genomic profiling of tumors was conducted through next-generation sequencing (NGS) via Roche-FoundationOne<sup>®</sup>.

**Results:** Mutations were found in APC (76.5%), TP53 (58.8%) and KRAS (52.9%). Only KRAS wild-type samples showed FBXW7. APC frameshift mutations and MLH1 splice variant were conversely significant correlated (7% v 93%,  $P = 0.014$ ). The median number of gene mutations reported was 6 (range 2–14). The TP53 mutation was associated most frequently with lung metastasis ( $P = 0.07$ ) and high tumor budding ( $P = 0.03$ ). Despite no statistical significance, lung recurrence, LVI/Pni, MSI and more than 6 genetic mutations were correlated to worse DFS and OS. Patients carried co-mutations of TP53-FBXW7 reported the worse DFS (4 v 14 months) and OS (4 v 65 months) compared to the other patients.

**Conclusion:** According to the present analysis, the setting of relapsed CRC emerges as one of the fields of greatest utility for NGS, looking at personalized cancer care.

**Keywords:** next-generation sequencing, NGS, colon cancer, stage II, biomarkers

## Introduction

Colorectal tumorigenesis is a multistep process that involves an accumulation of multiple, successive genetic alterations like chromosomal abnormalities, gene mutations, and epigenetic changes, that induces neoplastic transformation of the colic epithelium.<sup>1</sup> The most commonly genetic aberrations in colorectal cancer (CRC) are reported in APC (Adenomatous Polyposis Coli), TP53, RAS (rat sarcoma viral oncogene homolog) and PIK3CA (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) genes.<sup>2–6</sup> Some of these alterations, whose prognostic and predictive role is well established, are routinely used to make personalized treatment decisions in CRC.<sup>7</sup>

Approximately 80% of CRC presents as a localized or regional disease at diagnosis, but 35% of these patients develop a disease relapse, which in most cases (80%) occurs within the first 3 years of surgery.<sup>8</sup> The use of adjuvant chemotherapy, of which fluoropyrimidines are still the backbone, is associated with reduction of recurrence in 25% of patients with stage III

CRC,<sup>9</sup> while the absolute benefit in stage II CRC is only 3–4%.<sup>10–12</sup> In retrospective analyses, different genes were involved in the prognosis and prediction of response to chemotherapy in early-stage CRC, especially KRAS, BRAF, PIK3CA and p53.<sup>13–19</sup> A recent analysis of 130 patients with stage II–III CRC revealed that TYMS/KRAS/BRAF molecular profiling predicts survival after adjuvant chemotherapy.<sup>20</sup> The loss of heterozygosity at the TYMS locus on chromosome 18 showed a role in the genesis of resistance to fluoropyrimidine-based therapy.<sup>21</sup> APC mutations, associated with poor prognosis in 5-fluorouracil treated stage III colon cancers, have shown no correlation with disease-free survival in stage II colon cancers.<sup>22</sup> A role of the axis fibroblast growth factor 1/fibroblast growth factor receptor 3 (FGF1/FGFR-3) and overexpression of FGFR was recognized in tumour growth and metastatization.<sup>23,24</sup> Negative prognostic characteristics like right-sided tumors, microsatellite instability, BRAF mutations, lymph node metastases, and a higher tumor stage seem to be more frequently correlated with PTEN alterations that may lead to poor prognosis.<sup>25,26</sup> The predictive and prognostic role of PI3K/AKT pathway<sup>27,28</sup> and ERBB4 gene<sup>29–31</sup> in CRC is still debated. In this complex context, the identification of biomarkers through extensive molecular profiling of stage II CRC could provide more information about diagnosis, prognosis, but especially to identify those patients who will receive more benefit from 5-Fluorouracil (5FU)-based adjuvant chemotherapy.

On this basis, the aim of our study was to investigate comprehensive genomic profile of relapsed stage II CRC patients treated at our hospital. The secondary aim was to evaluate the possible association between these molecular alterations in respect to both survival outcomes and clinicopathological characteristics.

## Materials and Methods

### Study Design

This is a mono-institutional retrospective analysis that has examined formalin-fixed paraffin-embedded (FFPE) tissue blocks retrieved from surgical resection, from patients affected by r stage II CRC, who underwent surgery at Sant' Andrea Hospital in Rome from 2009 to 2015 and whose comprehensive genomic profiling of tumors was conducted through next-generation sequencing (NGS) via FoundationOne<sup>®</sup>.<sup>32,33</sup> The study enrolled 17 patients, who reported a relapse of disease, out of those 174 patients included in a previous analysis of stage II CRC<sup>34</sup> (Figure 1). Out of those 17 patients, 6 were affected by stage II CRC high risk according to NCCN guidelines definition.<sup>35</sup> All patients included in our study received follow up in our center, six of these received also adjuvant 5-fluorouracil-based chemotherapy due to the high risk of the tumor. Patient demographics and clinical characteristics were also examined.

To compare our results with validate literature data, we assumed data from cBioPortal for Cancer Genomics, that converts molecular profiling data from cancer tissues and cell lines in-to highly accessible genetic, epigenetic, gene expression, and proteomic events (<https://www.cbioportal.org>). Oncoprints (Figures 2–4) show mutational frequency of selected genes on The Cancer Genome Atlas colon adenocarcinoma (COAD TCGA) Cohorts, respectively in all population stage II CRCs, non-recurred and recurred CRCs. The first objective of our study was to explore genetic mutations, amplifications, or alterations in patients with relapsed stage II CRC. The secondary objectives were to correlate genomic profile to survival outcomes and clinicopathological characteristics.

All patients signed an informed consent for scientific research purpose at the first oncological visit. The study was conducted in accordance with the Declaration of Helsinki and the protocol approved by the institutional (Sant' Andrea Hospital, Sapienza University) ethical committee (N.3874\_2015).

### Determination of Gene Mutations/Next Generation Sequencing

Comprehensive genomic profiling was performed on DNA extracted from 17 formalin-fixed paraffin-embedded (FFPE) tissue sections from one block representative of the tumor using the FoundationOne (Foundation Medicine, Cambridge, USA). Foundation Medicine is a next-generation sequencing based in vitro diagnostic test that uses targeted high throughput hybridization-based capture technology for detection of substitutions, insertion and deletion alterations, and copy number alterations in 315 cancer-related genes plus introns from 28 genes often rearranged or altered in solid tumors. Furthermore, the assay provides information about Microsatellite status and tumor mutation burden (<https://www.foundationmedicine.com>). All NGS-results are also listed in [Supplementary Table](#). The gene panel list is listed in [Supplementary Table](#).

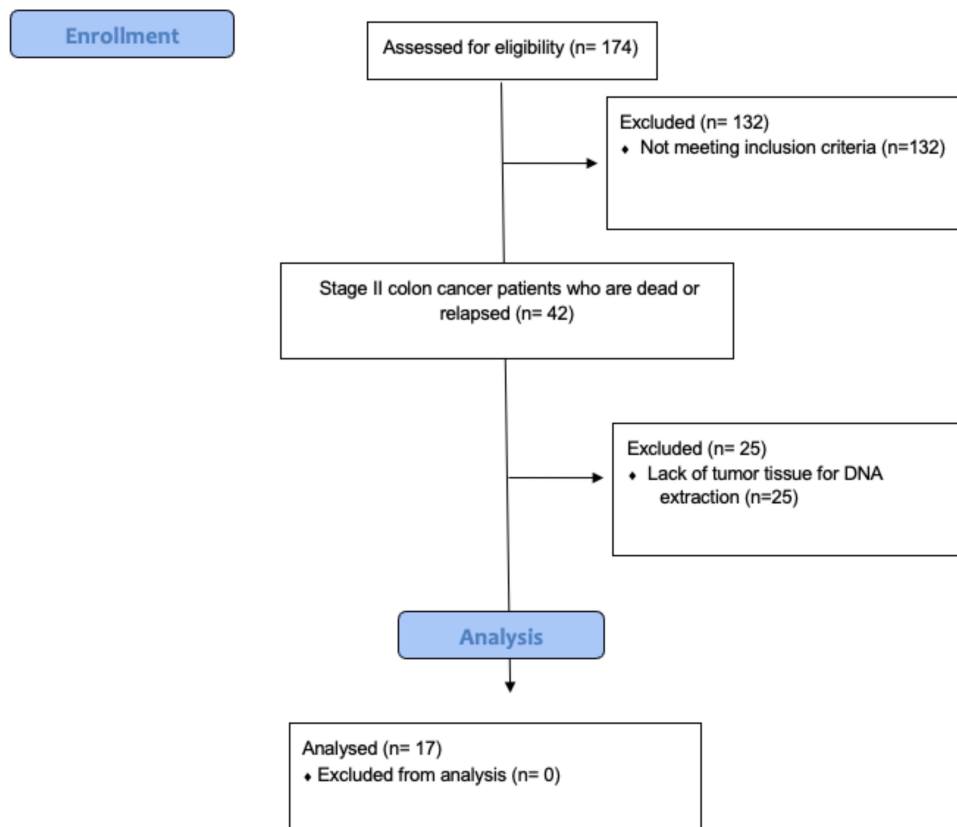


Figure 1 CONSORT 2010 flow diagram.

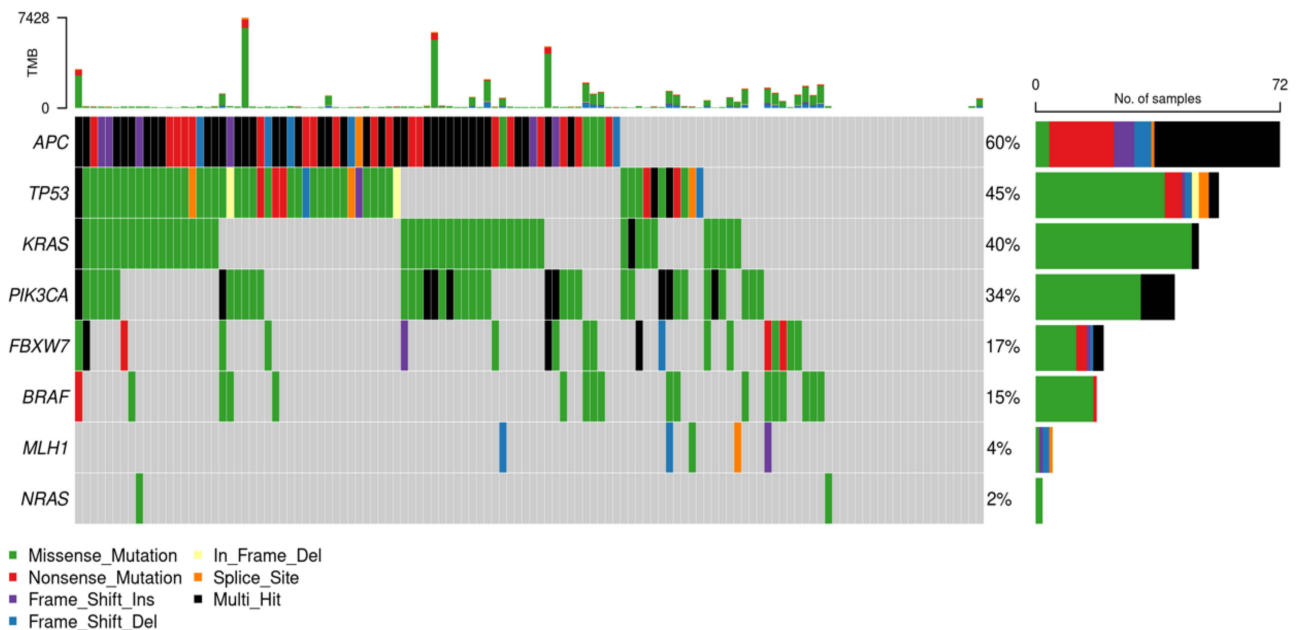
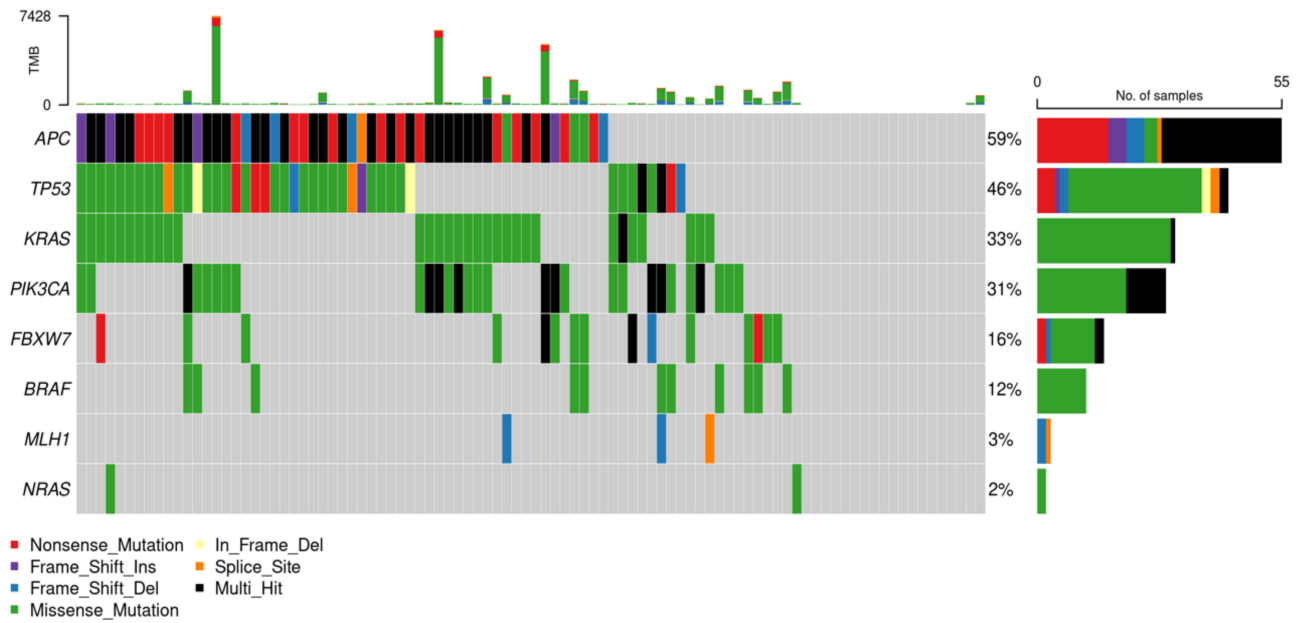


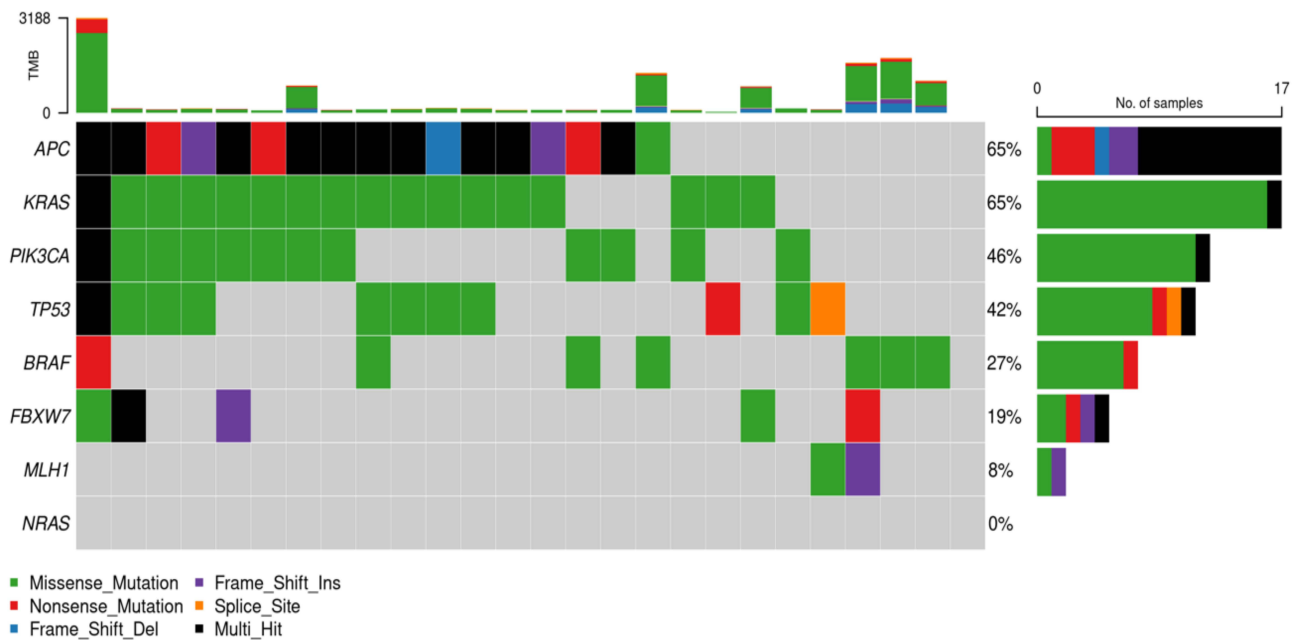
Figure 2 The Cancer Genome Atlas Colon Adenocarcinoma Cohorts (TCGA COAD) stage II CRCs.

### Statistical Analysis

Patient characteristics and gene mutation frequencies were described using descriptive analysis. Data are presented as number, number and percentage, or median and range. Pearson’s  $\chi^2$  test or Fisher’s exact test was used to evaluate



**Figure 3** The Cancer Genome Atlas Colon Adenocarcinoma Cohorts (TCGA COAD) stage II non recurred CRCs.



**Figure 4** The Cancer Genome Atlas Colon Adenocarcinoma Cohorts (TCGA COAD) stage II recurred CRCs.

associations between TP53, KRAS, PIK3CA, and FBXW7 mutations, and dMMR, CDX2, tumor budding and other clinicopathologic variables such as sidedness of primary tumor (right vs left), sites of metastases. Kaplan-Meier method was used for survival analysis. Correlation between clinicopathological characteristics and survival was done by Cox regression. Statistical calculations were performed using SPSS Statistics version 24 (SPSS, Inc., Chicago, IL, USA). P-values less than 0.05 were considered statistically significant.

## Results

### Clinicopathological Characteristics

A total of 174 consecutive patients with stage II colon cancer were screened, and those 17 (10%) patients with a relapse of disease within the first 5 years of follow up for colon cancer, were included in this study. Tissue blocks of primary tumor were available for all included patients. The median age of subjects was 71 years (range:43–84), most of them (14, 82%) were male, and affected with right-side colon cancer rather than left-side colon cancer (71% and 29%, respectively). Fourteen (82%) tumors were pT3, and only one case reported <12 lymph-nodes retrieval. The majority of cases were moderately differentiated tumors (65%), without lymphovascular invasion (67%) and with a low stage risk according to international clinical guidelines (65% and 35% was low and high stage risk, respectively). Out of 1 patient who reported a local recurrence, 15 patients experienced a distant recurrence, especially in liver (35%) and lung (35%). Other patient and tumor characteristics are summarized in [Table 1](#).

### Comparison with Clinical Factors

NGS analysis was carried out in all patients and median exon coverage is >500x for all samples. All NGS-results are also listed in [Supplementary Table](#). Genomic profile was different and heterogenous among the study patients ([Table 2](#)). Most of the patients reported mutations in the following genes: APC (76.5%), TP53 (58.8%) and KRAS (52.9%). Gene mutations frequency was represented in [Figure 2](#). Among the KRAS gene mutations, 8 (88%) had mutation in exon 2 (G12D, G13D), 1 (12%) in exon 3 (K117N). PIK3CA mutations were identified in 5 (29.4%) tumors. All of these had mutation in exon 9 (E545K, E542Q, R88Q, and R849\*). FBXW7 as well as MLH1 were both reported in 3 cases (17.6%). FBXW7 were found only in KRAS wild-type samples. MLH1 splice variant and APC frameshift mutations were conversely significant reported (7% v 93%,  $P = 0.014$ ). No other significant relationship was found. However, TP53-KRAS, TP53-PIK3CA and TP53-FBXW7 mutations were co-occurrent in 5 (29%), 3 (18%) and 2 (12%) patients, respectively. Only 1 patient carried KRAS-PIK3CA co-mutations.

Additional mutations were also detected in 11.8% of cases (ARID2, BRAF, CIC, KMT2C (MLL3), LRP1B, NRAS, PTEN, RNF43, SMAD4 and SOX9TNNB1, NRAS, and SMAD4) and other 42 genes at lesser frequency. Overall, the median number of gene mutations reported was 6 (range 2–14).

Patients who carried a TP53 mutation, developed disease recurrence more frequently in the lung than liver or other metastatic sites. (55% v 27% v 18%,  $P = 0.07$ ). No other significant correlation was found between genetic mutations and clinicopathological factors.

In order to integrate the gene mutations panel with the potential molecular and biological consequences involved in CRC relapse, we performed a STRING network analysis. STRING is a database of known and predicted protein-protein interactions that includes direct and indirect associations.<sup>36</sup> We included in STRING analysis, both genes that presented mutation incidence > 18% in our analysis - APC, TP53, KRAS, PIK3CA, FBXW7, MLH1 – ([Figure 5](#)), and the others most important genes involved in colorectal cancer process including BRAF and NRAS. On the basis of this analysis, we can hypothesize that other gene mutations in addition to those we usually use in CRC management (KRAS, NRAS, BRAF), could figure out as novel target to improve the landscape of relapsed CRC treatment ([Figure 6](#)). These possible future targets are represented by genes involved in mechanisms of cellular senescence and aging, immune response, intracellular signal transduction mediated by members of the RAS superfamily of proteins. Several are the evidence of a role of tumor microenvironment in treatment response and relapse such as the interplay between cancer cells and cancer-associated fibroblasts (CAFs) that has been proven to promote colorectal carcinogenesis.

### Prevalence of dMMR/MSI-H, CDX2 and Tumor Budding

Microsatellite instability was reported in 4 (18%) patients, 3 (67%) of whom carrying MLH1 mutation. Only 1 patient was CDX2 negative. This single patient was carrier of FBXW7 mutation ( $P = 0.02$ ) Tumor budding was equally represented: high and low was both reported in 8 (50%) patients, and it was not examined in 1 case. Tumor budding high was frequently associated with TP53 mutation (88% v 12%,  $P = 0.03$ ). Contrary, MLH1 mutation was present only in samples with tumor budding low ( $P = 0.05$ ). Accordingly, tumor budding high was not reported in MSI tumors ( $P = 0.05$ ).

**Table I** Clinicopathologic Features of Patients

<b>Clinicopathologic Features (Valid Cases and Percentages)</b>			
		<b>N.</b>	<b>%</b>
<b>Total</b>		17	100
<b>Follow up time</b>			
<b>Median (range min-max)</b>	60 (4–107)		
<b>Age</b>			
<b>Median (range min-max)</b>	71 (43–84)		
<b>&lt;70 years</b>		7	41
<b>≥70 years</b>		10	59
<b>Sex</b>			
<b>Male</b>		14	82
<b>Female</b>		3	18
<b>Tumour location</b>			
<b>Right-side colon</b>		12	71
<b>Left-side colon</b>		5	29
<b>pT stage</b>			
<b>T3</b>		14	82
<b>T4</b>		3	17
<b>N. of Lymph-node retrieved</b>			
<b>&lt;12</b>		1	6
<b>≥12</b>		16	94
<b>Lymphovascular/Perineural invasion (n=14)</b>			
<b>Not</b>		7	67
<b>Yes</b>		4	35
<b>Tumour differentiation (grading)</b>			
<b>G2</b>		11	65
<b>G3</b>		6	35
<b>Histopathological Stage risk</b>			
<b>Low</b>		9	53
<b>High</b>		8	47
<b>Relapse</b>			
<b>Oligometastatic</b>		8	47
<b>Multiple metastases</b>		9	53
<b>Local Recurrence</b>		1	6
<b>Distant recurrence</b>		14	82
<b>Liver</b>		5	36
<b>Lung</b>		5	36
<b>Bone</b>		2	14
<b>Lymph-nodes</b>		2	14
<b>Abdominal wall</b>		1	7
<b>Microsatellite Instability</b>			
<b>MSS</b>		14	82
<b>MSI-H (BRAF mut)</b>		3 (3)	18 (100)

(Continued)

**Table 1** (Continued).

<b>Clinicopathologic Features (Valid Cases and Percentages)</b>		
	<b>N.</b>	<b>%</b>
<b>CDX2 expression</b>		
<b>Positive</b>	16	94
<b>Negative</b>	1	6
<b>Tumor budding (n=16)</b>		
<b>Low/absent</b>	8	50
<b>High</b>	8	50
<b>Adjuvant CT</b>		
<b>Nil</b>	11	65
<b>Oral capecitabine</b>	3	18
<b>FOLFOX</b>	3	18

**Abbreviations:** MSS, microsatellite status stable; MSI-H, microsatellite status instable-high; BRAF mut, BRAF mutation; CT, chemotherapy; Nil, not done.

## Survival Analysis

With a median follow up of 53 months (range 4–107), median DFS and OS were 14 and 65 months, respectively. None of variables analyzed were significantly associated to survival. However, patients with high stage risk, lung recurrence, pT4 tumor, LVI/Pni, MSI, FBXW7 mutant carriers and with more than 6 genetic mutations showed the worse DFS (Table 3). Patients with age >70, right colon cancer, lung recurrence, LVI/Pni, grade G3, MSI, carriers of PIK3CA, FBXW7 or MLH1 mutations and with more than 6 genetic mutations showed the worse OS. Moreover, patients carried co-mutations of TP53-FBXW7 reported the worse DFS and OS compared to the other patients studied (DFS: 4 v 14 months; OS: 4 v 65 months).

## Discussion

Stage II CRC is characterized by a good prognosis, with 5-year overall survival of 70–80% after surgery,<sup>37</sup> but about 20% of these patients develop a tumor relapse.<sup>10,38</sup> The adjunct of a 5-fluorouracil-based adjuvant chemotherapy provide an absolute benefit of 3–4% only<sup>10–12,39</sup> and the current international clinical guidelines ([https://www.nccn.org/professionals/physician\\_gls/pdf/colon.pdf](https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf)) seems to be insufficient to identify the “real” high risk stage II CRC. The identification of biomarkers through molecular profiling of tumors, could help clinicians mostly to identify those patients who will receive more benefit from adjuvant chemotherapy. With this aim, we conducted a mono-institutional retrospective analysis of 174 patients affected by stage II CRC who underwent surgery at Sant’ Andrea Hospital. We enrolled only those seventeen (10%) patients who reported a relapse of disease, whose comprehensive genomic profiling of tumor through NGS (FoundationOne<sup>®</sup>) was conducted. The possible association in respect to clinicopathological characteristics and survival outcomes was explored.

According to literature data the recurrence rate of our population was about 10%.<sup>8</sup> Lymphovascular and perineural invasion by cancer cells, although very promising as prognostic variables, have proved difficult to standardize due to technical problems inherent in the visual analysis and the subjective definition of these features.<sup>40</sup> Nevertheless, there are data in literature about association between poor histological differentiation and advanced T stage and the presence of lymph node involvement.<sup>41</sup> according to this knowledge, the pathological characteristics of stage II CRC of our study, showed that 65% of cases were moderately differentiated tumors, and 33% showed lymphovascular invasion.

Six patients received adjuvant 5-fluorouracil-based chemotherapy in relation to the risk. As previously reported, stage II CRC receive a small benefit with adjuvant chemotherapy in QUASAR trial<sup>32</sup> and in the MOSAIC trial, there was no difference in DFS in stage II patients who received chemotherapy and those who did not.<sup>42</sup> Nowadays, guidelines recommended adjuvant chemotherapy in “high-risk” stage II patients, which include (exclusive for those cancer that are

**Table 2** Genomic Profile and Clinicopathological Characteristics of Relapsed CRC Patients

Patient Number	Sex	Age	Number of Genes	Genes	Sidedness CRC	Site of Recurrence	MMR Status	Risk	Adj CT	mDFS	mOS
01	M	71	14	APC, TP53 KRASMLH1 ARID2, CIC KMT2C SOX9, MSH2 CDKN1B EPHA5 MED12 PTCH1 RUNX1	R	Lung	MSI	High	None	12	19
02	F	77	10	APC, TP53 KRAS KMT2C ASXL1 CDK8, IRS2 CEBPA EGF14, FLT3	R	Lung	MSS	High	None	10	65
03	F	75	10	PIK3CA MLH1 PTEN LRPBI RNF43 SOX9, ATM CTNNAI FANCA	R	Liver	MSI	Low	Cape	16	46
04	M	69	10	APC, TP53 NRAS AURKA BCL2LI GNAS, TOPI KEL, SRC ZNF217	R	Liver	MSS	Low	None	40	57
05	M	76	7	APC, TP53 PIK3CA FBXW7 ARID2 EGFR, EZH2	R	Lung	MSS	Low	None	4	4
06	M	84	7	APC, TP53 PIK3CA KMT2C FAMI23B FGFR1, NFI	R	Lung	MSS	Low	None	14	49
07	M	69	6	AP, BRAF FBXW7 SMAD4 AKT1, GRM3	R	Lymph-nodes	MSI	Low	Folfox	11	17

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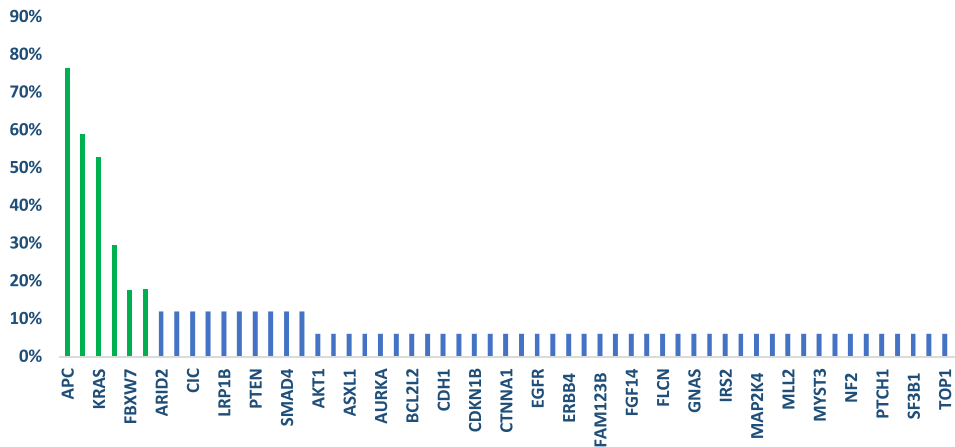
Table 2 (Continued).

Patient Number	Sex	Age	Number of Genes	Genes	Sidedness CRC	Site of Recurrence	MMR Status	Risk	Adj CT	mDFS	mOS
08	M	70	6	APC, TP53 BCL2L1 ERBB4 SPTAI, SRC	L	Bone	MSS	High	Folfox	25	30
09	M	74	6	APC, TP53 KRAS, FLCN BCL2L2 MYST3	R	Liver, lymphnodes	MSS	Low	None	45	65
10	M	58	6	KRAS, MLL2 MLH1, CIC RNF43 CTNNB1	L	Local	MSI	Low	Cape	54	63
11	M	82	5	APC, KRAS LRP1B ERBB4 MAP2K4	R	Bone, lymphnodes	MSS	High	None	14	53
12	F	75	5	APC, KRAS BRCA2 NF2, SF3B1	L	Liver	MSS	Low	None	14	77
13	M	68	5	APC, TP53 FBXW7 NRAS CHD1	L	Lung	MSS	High	Folfox	23	54
14	M	69	5	APC, TP53 PIK3CA PTEN ARID1A	R	Liver	MSS	Low	None	30	37
15	M	62	5	APC, TP53 KRAS SMAD4 PIK3CG	R	Abdominal wall	MSS	Low	Cape	31	81
16	M	43	3	APC, KRAS PIK3CA	L	Liver	MSS	Low	None	10	107
17	M	75	2	TP53, KRAS	R	Lung	MSS	High	None	4	21

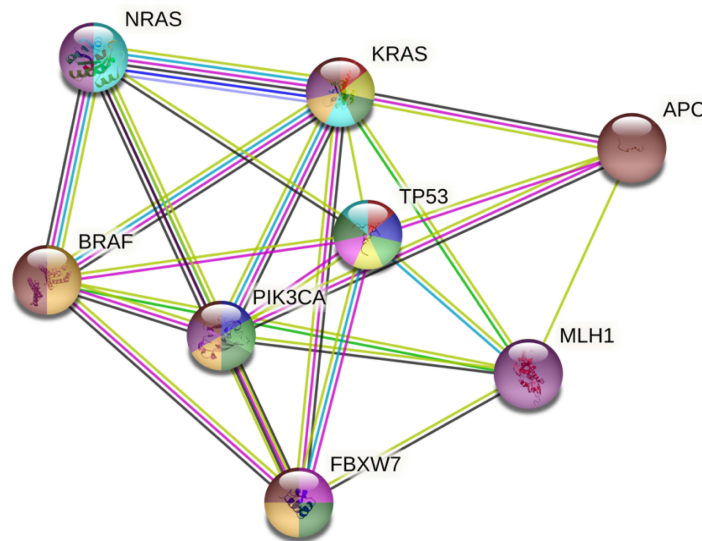
**Abbreviations:** M, male; F, female; CRC, colorectal cancer; R, right; L, left; MMR, mismatch repair; MSS, microsatellite status stable; MSI, microsatellite status instable; Adj CT, adjuvant chemotherapy; mDFS, median disease-free survival; mOS, median overall survival.

MSI-H) poorly differentiated/undifferentiated histology, lymphatic/vascular invasion, bowel obstruction, <12 lymph node sampling, peri-neural invasion, perforation, or positive margins ([https://www.nccn.org/professionals/physician\\_gls/pdf/colon.pdf](https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf)).

In literature, there is the evidence of a predictive value of several mutant genes, but most previous studies combined all stages of CRC.<sup>43</sup> With the aim of clarifying the molecular effects, we conducted a study only in relapsed stage II CRC, with the small sample size limitation, compared to more than 300 genes explored by NGS.



**Figure 5** Frequency and representation of gene mutations in cohort study. Vertical axis: frequency of mutations. Horizontal axis: genes.



	<i>Biological Process</i>	<i>Strenght *</i>	<i>False discovery rate #</i>
●	Positive regulation of cell aging	2.51	0.0164
●	Regulation of fibroblast apoptotic process	2.46	0.0164
●	Negative regulation of macroautophagy	2.17	0.0344
●	Regulation of cellular senescence	2.06	0.0483
●	Regulation of autophagy of mitochondrion	2.06	0.0483
●	Regulation of neuron apoptotic process	1.66	0.0100
●	RAS protein signal transduction	1.5	0.0347
●	Positive regulation of protein kinase activity	1.25	0.0203
●	Positive regulation of immune response	1.21	0.0261
●	Regulation of protein kinase activity	1.18	0.0135

**Figure 6** STRING network analysis of relevant gene mutations (N=17 patients). \*Log10 (observed/expected). This measure describes how large the enrichment effect is. It's the ratio between i) the number of proteins in your network that are annotated with a term and ii) the number of proteins that we expect to be annotated with this term in a random network of the same size. #This measure describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

**Notes:** Color of edges represents protein-protein associations. Known interactions: pink: experimentally determined; turquoise: from curated databases. Predicted interactions: green: gene neighborhood; red: gene fusions; blue: gene co-occurrence. Others interactions: yellow: text mining; black: co-expression; light blue: protein homology. Color of balls represents biological process in which genes are involved as showed in figure above.

Complete spectrum of gene mutations is represented in [Supplementary Table](#) and their percentages distribution in our cohort of patients in [Figure 2](#). The most of mutations involves genes whose prognostic and predictive role is widely reported in the literature:<sup>13,14,17,18</sup> 76.5% of our patients showed APC mutations, 58.8% TP53, 52.9% KRAS and 29.4% PIK3CA. The involving of these genes is in line with the Cancer Genome Atlas colon adenocarcinoma (TCGA COAD) cohort. Mutations in APC, a tumor suppressor gene with an important role in the WNT signaling pathway, intercellular adhesion, cytoskeleton stabilization, cell cycle regulation, and apoptosis,<sup>44–48</sup> appear to play a role in promoting tumorigenesis;<sup>49,50</sup> while mutations of p53 tumor suppressor gene are among the commonest genetic alterations in all cancers. As we know KRAS and BRAF mutations, especially for left-sided and rectal tumors were associated with poor prognosis<sup>13–16</sup> while PIK3CA mutations was associated with increased risk of local recurrence.<sup>17</sup>

FBXW7 is a critical tumor suppressor gene that encodes the substrate recognition components of SKP1-Cullin1-F-box protein ubiquitin E3 ligase complexes,<sup>51,52</sup> that negatively regulate the intracellular abundance of various key oncogenic proteins. Therefore, the loss of FBXW7 function results in accumulation of its substrates, which leads to oncogenesis and progression of multiple cancers, including CRC.<sup>53</sup> Clinical studies reported that the low expression level of FBXW7 was related to a poor prognosis.<sup>54,55</sup> The frequency of this alterations (17.6%) in our study is slightly higher than the previously reported rates.<sup>56–59</sup> Noteworthy, we found FBXW7 mutations only in KRAS wild-type samples while in previous reports KRAS was frequently found as a concomitant mutation, especially in patients with colorectal cancer (until 86%).<sup>60,61</sup>

About co-occurring mutations, we reported TP53-PIK3CA, TP53-FBXW7 and TP53-KRAS in 3 (18%), 2 (12%) and 5 (29%) patients, respectively. The last one is an uncommon combination: TP53 and KRAS mutations were rarely found together in the same tumor, suggesting different genetic pathways leading to tumor formation. The low number of tumors containing mutations in both genes could suggest that this phenotype may have arisen by chance.<sup>62,63</sup> Indeed, we excluded by our analysis, patients with familiar history of CRC. Moreover, co-altered RAS/BRAF/TP53 resulted as an independent factor for mortality and was associated with worse OS in patients with liver and lung metastases. These tumors were significantly more likely to involve extrahepatic metastatic sites:<sup>64</sup> to confirm this, four of our five total patients harboring this co-alteration, have metastasized in lung (3 patients) and abdominal wall (1 patient), although no significant correlation was found between these genetic mutations and clinicopathological factors.

An early event in colorectal carcinogenesis<sup>65,66</sup> is the promoter hypermethylation of the mismatch repair gene Human Mut-L Homologue 1 (MLH1), frequently associated with microsatellite instability and BRAF mutations in CRC.<sup>67–71</sup> Previous studies observed that microsatellite instable tumors may harbor less aberrations in KRAS, TP53 or APC, suggesting that these tumors develop through a distinct pathway.<sup>72–75</sup> Vogel et al<sup>76</sup> confirmed that MLH1 hypermethylation was less common in tumors with APC mutations or KRAS mutations; and our data showed that MLH1 splice variant and APC frameshift mutations were conversely significant correlated (7% v 93%). Instead, microsatellite instability was reported in 4 (18%) patients, 3 (67%) of whom carrying MLH1 mutation. This is in line with the observed high correlation between MLH1 methylation and microsatellite instability in the colorectum<sup>68–70</sup> and its inverse relationship with mutations in other key genes involved in colorectal carcinogenesis.<sup>72–75</sup>

The idea that the tumor biology and key mutations might differ between various sites of metastatic manifestation is well established:<sup>77</sup> p53 mutations promoted CRC metastasis, including lymphatic and distant metastases, in European and American populations.<sup>78</sup> A complete mutational status of CRC lung metastases and corresponding primary tumors by NGS, with a concordance of 83.5%, founded mutations in TP53 in 57.4% of both primary tumors and corresponding lung metastasis.<sup>79</sup> In our work patients who carried a TP53 mutation, developed disease recurrence more frequently in the lung (55% v 27 v 18,  $P = 0.07$ ).

In addition to the effects of single gene mutations, the median number of gene mutations, which we know to be related with the stage,<sup>80</sup> was also evaluated, and we reported a median number of 6 mutations (range 2–14) in our population.

Previous studies indicated that CDX2 negative tumors are often associated with several adverse prognostic variables such as advanced stage, poor differentiation, vascular invasion, BRAF mutation, and CIMP-positive status.<sup>81–84</sup> Loss of CDX2 expression has been reported in several colorectal cancers and cancer cell lines with a potential inverse correlation between CDX2 levels and tumor stage;<sup>85</sup> actually, the combination of the T stage with the CDX2 expression status seems

to have better prognostic prediction value in CRC from stage I to stage III.<sup>86</sup> Only one patient affected by stage II CRC was CDX2 negative, moreover was carrier of FBXW7 mutation ( $P = 0.02$ ) and MSI. These reports indicated the involvement of FBXW7 in the development and regulation of cellular differentiation by targeting multiple substrates for degradation and can be explained by the FBXW7-mediated inhibition of CDX2 expression, that also could lead to reduced CDX2 transactivation and growth arrest of colon cancer cells.<sup>85</sup> Indeed, overexpression of FBXW7 in colon cancer cells led to a reduction of CDX2 protein levels.

MSI-H, common among stage II (20%) and III (12%)<sup>87</sup> confers a favorable prognosis in patients with localized disease<sup>88,89</sup> and results associated with CDX2 negative status ( $P < 0.001$ ) and vice versa.<sup>34</sup>

In MSI patients, tumor budding high was not reported ( $P = 0.05$ ) and accordingly MLH1 mutation was present only in samples with tumor budding low ( $P = 0.05$ ). Tumors with high-level MSI, seem to have very low rates or no tumor budding:<sup>90</sup> indeed, the invasive front of colorectal cancers can be thought as a dynamic interface of pro- and anti- tumor factors composed primarily of cytotoxic T lymphocytes, to protect vascular and lymphatic channels from invasion by tumor buds<sup>91</sup> and MSI-H colorectal cancers exemplify this attacker/defender model and highlight a pro-immunogenic phenotype.<sup>92</sup> Accordingly, in the study by Romiti et al, only 16% of populations with high tumor budding presented microsatellite instability.<sup>34</sup>

With a median follow up of 53 months (range 4–107), median DFS and OS were 14 and 65 months, respectively. Despite none of variables analyzed resulted significantly correlated to survival, patients with high stage risk, lung recurrence, pT4 tumor, LVI/Pni, MSI, FBXW7 mutant carriers showed worse DFS. (Table 3) Many of these features are reported in literature as prognostic factors in term of recurrence after surgery.<sup>93–96</sup> Perineural invasion, because of its correlation with depth of tumor invasion and lymphovascular invasion, was defined as a prognostic factor for the survival of patients with stage II CRC.<sup>97</sup> In a recent work by Shinto et al<sup>98</sup> pT4 appeared as an independent poor prognostic factor, specifically in stage II CRC. The substantiation of MSI status determination in stage II CRC patients may be controversial: on one side we have NCCN recommendations, but on the other side we should consider the results of the meta-analysis by Des Guetz et al<sup>99</sup> that observed no statistically significant difference in patient survival in terms of Regression-Free Survival or OS irrespective of adjuvant chemotherapy implementation for the MSI-H patients.

In terms of OS, worse outcome was demonstrated by patients harboring these characteristics: age  $>70$ , right colon cancer, lung recurrence, LVI/Pni, grade G3, MSI, carriers of PIK3CA, FBXW7 or MLH1 mutations. In patients with pulmonary metastasis, the frequency of KRAS mutations was significantly higher,<sup>100</sup> along with those of APC and TP53,<sup>101</sup> mutations that we could define as signatures associated with poor prognosis. As reported above, the role of the MSI status as a predictive marker remains controversial,<sup>102</sup> so this molecular signature could allow for stage II dismemberment, more precisely identifying patients at high and low risk of relapse.

A retrospective analysis of 93 CRC cases has showed in patients with low FBXW7 expression, a worse 5-year OS than those in the high expression group, to demonstrate the prognostic role of FBXW7.<sup>47</sup> Furthermore, FBXW7 missense mutations are associated with PIK3CA mutations and with a shorter OS.<sup>52</sup> Loss of function of this gene seems to be implicated in anti-EGFR resistance,<sup>103</sup> in increased resistance to paclitaxel,<sup>104</sup> regorafenib,<sup>105</sup> 5-fluorouracil,<sup>106</sup> or oxaliplatin.<sup>107</sup> This implication of FBXW7 status in chemoresistance, could suggest the potential of FBXW7-targeted therapy, as demonstrated in other field of tumor disease.<sup>108</sup>

Patients with more than 6 genetic mutations and carried co-mutations of TP53-FBXW7 reported the worse DFS and OS compared to the other patients studied. Co-deletion of both FBXW7 and p53 causes highly penetrant, aggressive, and metastatic adenocarcinomas that frequently metastasized to lymph nodes and liver and often exhibited chromosomal instability,<sup>109</sup> (Supplementary Figures 1 and 2).

There are several limitations to this study. First, the retrospective nature of this analysis, the small sample size of our populations and the lack of a control group of patients affected by stage II CRC not relapsed. Furthermore, NGS was conducted only in formalin-fixed paraffin-embedded (FFPE) tissue blocks retrieved from surgical resection; we did not provide a complete mutational status of corresponding metastases however in colon cancer the concordance between primary tumors and metastases was near to 90%.<sup>64</sup> Nevertheless, we recognize that the characteristics of our study do not allow to draw strict conclusion, but we can make speculative assessments about gene mutations involved in relapse of

**Table 3** Univariate Analysis for Disease Free Survival (DFS) and Overall Survival (OS)

Variables	Disease Free Survival		Overall Survival	
	Median Months	HR (95% CI)	Median Months	HR (95% CI)
Sex F v M	14 v 14	2.0 (0.5–7.9)	65 v 81	1.0 (0.2–5.3)
Age >70 v ≤70	14 v 30	2.1 (0.7–6.0)	46 v 81	2.3 (0.5–11.6)
Colon right v left	14 v 23	1.5 (0.5–4.8)	46 v NR	4.7 (0.6–37.5)
Risk high v low	12 v 16	2.4 (0.7–7.6)	30 v 81	2.2 (0.5–9.0)
Recurrence lung v liver v other	10 v 16 v 25	3.9 (1.0–14.4)	21 v NR v 81	3.9 (0.6–22.2)
Tumor pT4 v p3	10 v 14	2.8 (0.7–10.7)	65 v 81	1.5 (0.3–7.3)
LVI/Pnl yes v not	10 v 25	4.1 (0.8–18.9)	21 v 46	1.5 (0.3–6.9)
Grade G3 v G2	14 v 14	1.3 (0.4–3.7)	30 v 81	1.7 (0.4–7.5)
Microsatellite MSI v MSS	12 v 14	1.4 (0.4–5.2)	19 v 81	3.2 (0.7–14.6)
Tumor budding high v low	14 v 14	1.7 (0.6–5.1)	65 v 46	1.3 (0.3–4.7)
APC mut v wt	14 v 16	1.9 (0.4–8.6)	81 v 46	0.6 (0.1–2.8)
TP53 mut v wt	23 v 14	1.0 (0.4–3.0)	65 v NR	2.3 (0.5–11.3)
KRAS mut v wt	14 v 16	1.2 (0.4–3.5)	81 v 37	0.3 (0.1–1.5)
PIK3CA mut v wt	14 v 14	1.9 (0.6–6.0)	46 v 65	1.2 (0.3–4.7)
FBXW7 mut v wt	11 v 14	2.6 (0.7–9.9)	17 v 81	3.7 (0.7–19.9)
MLH1 mut v wt	16 v 14	0.5 (0.1–2.1)	46 v 81	1.9 (0.4–9.9)
TP53-KRAS mut v wt	12 v 14	1.0 (0.4–3.2)	65 v NR	1.5 (0.4–6.1)
TP53-PIK3CA mut v wt	14 v 14	1.5 (0.4–5.6)	37 v 81	2.5 (0.5–12.7)
TP53-FBXW7 mut v wt	4 v 14	2.1 (0.4–9.7)	4 v 65	1.9 (0.2–15.8)
N. genetic mutations >6 v ≥6	12 v 23	1.7 (0.6–4.9)	46 v 81	2.2 (0.5–9.0)

Abbreviations: M, male; F, female; mut, mutate; wt, wild type.

CRC. Our study showed how targeting specific molecular pathways and gene mutations has become a critical step in improving treatment options and increasing patient survival. Hence, the use of NGS in relapsed CRC should be widespread in clinical practice since the early stage of disease.

## Conclusion

Our retrospective work supports a deep molecular characterization of stage II CRC as a key element for optimal patient's management. Uniform guidelines to detect and classify variants, to interpret and report results should be recommended and adopted.<sup>110</sup> According to our analysis, the setting of relapsed CRC emerges like one of the fields of greatest utility for NGS, in clinical practice. Although the clinical implications of many genetic aberrations in CRC are still unclear, despite the extensive scientific research that has been conducted, the aim of this study is to emphasize the inclusion of genomic data into daily patient care and clinical guidelines. However, further researches are required to clarify the methodology to identify novel and reliable biomarkers and to help clinicians in treatment decisions in early stage CRC.

## Disclosure

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