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Less frequently mutated genes in colorectal cancer: evidences from next-generation sequencing of 653 routine cases

Umberto Malapelle,[1] Pasquale Pisapia,[1] Roberta Sgariglia,[1] Elena Vigliar,[1] Maria Biglietto,[2] Chiara Carlomagno,[3] Giuseppe Giuffrè,[4] Claudio Bellevicine,[1] Giancarlo Troncone[1]

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[1]Department of Public Health, University of Naples Federico II, Naples, Italy
[2]Department of Oncology, AORN Cardarelli, Naples, Italy
[3]Department of Surgical and Clinical Medicine, University of Naples Federico II, Naples, Italy
[4]Department of "Patologia Umana dell'Adulto e dell'età evolutiva, G.Barresi", University of Messina, Messina, Italy

Correspondence to
Professor Giancarlo Troncone, Department of Public Health University of Naples Federico II, via Sergio Pansini 5, Naples I-80131, Italy; giancarlo.troncone@unina.it

UM and PP contributed equally.

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ABSTRACT

Aims The incidence of *RAS/RAF/PI3KA* and *TP53* gene mutations in colorectal cancer (CRC) is well established. Less information, however, is available on other components of the CRC genomic landscape, which are potential CRC prognostic/predictive markers.

Methods Following a previous validation study, ion-semiconductor next-generation sequencing (NGS) was employed to process 653 routine CRC samples by a multiplex PCR targeting 91 hotspot regions in 22 CRC significant genes.

Results A total of 796 somatic mutations in 499 (76.4%) tumours were detected. Besides *RAS/RAF/PI3KA* and *TP53*, other 12 genes showed at least one mutation including *FBXW7* (6%), *PTEN* (2.8%), *SMAD4* (2.1%), *EGFR* (1.2%), *CTNNB1* (1.1%), *AKT1* (0.9%), *STK11* (0.8%), *ERBB2* (0.6%), *ERBB4* (0.6%), *ALK* (0.2%), *MAP2K1* (0.2%) and *NOTCH1* (0.2%).

Conclusions In a routine diagnostic setting, NGS had the potential to generate robust and comprehensive genetic information also including less frequently mutated genes potentially relevant for prognostic assessments or for actionable treatments.

INTRODUCTION

Antiepidermal growth factor receptor (*EGFR*) therapy is not effective in patients with metastatic colorectal cancer (CRC) harbouring mutations at codons 12 and 13 in *KRAS* exon 2.[1] More recent evidences showed that the so-called expanded *RAS* mutations (exon 3 and exon 4 of *KRAS* and exons 2, 3 and 4 of *NRAS*) also have negative predictive value.[2] The extension of community *KRAS* testing to all *RAS* mutations favoured the implementation of multitarget testing methodologies. Next-generation sequencing (NGS), matched with multiplex capture of targeted gene regions and analysed by bioinformatics tools, enables the simultaneous detection of multiple mutations in multiple genes. The development of affordable benchtop sequencers, such as the Ion Torrent Personal Genome Machine (PGM; Life Technologies, Carlsbad), and of relatively small, focused gene panels, such as the Ion AmpliSeq Colon and Lung Cancer Panel,[3] enabled our laboratory to adopt NGS as a stand-alone diagnostic test to genotype *KRAS NRAS* and *BRAF*.[4] In a previous validation study, all point mutations detected in these genes by Sanger sequencing were also correctly identified by NGS.[4] The latter, however, proved to be more sensitive, and, remarkably, less costly.[4]

NGS may also identify rarer patient-specific somatic mutations. The latter are of unclear significance, as their incidence rates have not been established with certainty. In fact, while there is a wealth of data regarding *RAS/RAF/PI3KA* and *TP53* gene mutations, the information on less frequently mutated genes is mostly derived by the genomic scale analysis of a limited number of CRC samples.[5] Conversely, in its daily diagnostic practice, our laboratory, an Italian accredited reference centre for *RAS* testing, has generated a large database of CRC samples sequenced with the PGM/Colon Lung Cancer Panel, whose interrogation can be useful to better define the incidence rate of rare mutations. Thus, besides *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53* alterations, this paper focuses on mutations occurring in other receptor tyrosine kinase (RTK) genes (*ALK*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *MET*, *DDR2*), in RTK signalling genes (*AKT1*, *PTEN*, *MAP2K1*, *STK11*) and in other well-known cancer-related genes (*NOTCH1*, *CTNNB1*, *SMAD4*, *FBXW7*).

METHODS

Patients and samples

This study includes a series of 653 CRC tissue samples (398 men and 255 women) referred from 18 institutions located all over South Italy between January 2014 and March 2015. Mean patient age was 66.8 years (range, 29–96 years). Following current international guidelines, one single tumour sample was tested for each patient.[6]

NGS analysis

Tumour cell enrichment, DNA extraction and NGS analysis on the Ion Torrent PGM by using the AmpliSeq Colon and Lung Cancer panel were performed, as previously described,[4] and detailed in online supplementary information (file 1). The Torrent Suite V.4.0 analysis pipeline was used to assess the sequencing data and to perform adapter trimming, alignment QC and base calling. Single-nucleotide polymorphisms, insertions and deletions (del) were identified using a Torrent Variant Caller plug-in (V.4.0-r76860), optimised for low-frequency variants assessment. The criteria for evaluation of any variant as reportable were the following: minimum coverage depth of 100×, minimum variant frequency of 5% and confirmation by the Integrative Genomics Viewer visual inspection. Sequence variants, deemed real and reportable

Table 1 Twenty-two multiple gene mutation analysis by the Ion Torrent AmpliSeq Colon and Lung Cancer Panel in routine samples of colorectal cancer

Total cases analysed	n=653
Wild type in all 22 gene analysed	n=154 (23.6%)
Mutated at ≥1 of 22 genes analysed	n=499 (76.4%)
Total mutations	n=796
Mutated genes	17/22

by criteria listed above, were further assessed by the ClinVar Database (http://www.ncbi.nlm.nih.gov/clinvar/, last accessed 30 November 2015) for classifying a genetic alteration as germline or somatic.

RESULTS

One or more gene mutations were detected in 499/653 (76.4%) tumours in 17 of the 22 genes included in the panel (table 1), for a total of 796 mutations that are listed in online supplementary information (file 2). A representative case is reported in figure 1. Only three genes (*DDR2*, *FGFR1* and *FGFR2)* did not harbour any alteration, while two genes *(FGFR3* and *MET*) only harboured germline variants as reported in online supplementary information (file 3). Single mutations were found in 274 patients (41.9%), double mutations in 177 patients (27.1%) and 3 or more mutations were found in 48 patients (7.4%). Coexisting mutations in different genes are reported in online supplementary table S1.

Mutations occurred in *TP53* (n=240; 38.8%), *KRAS* (n=247; 37.8%), *NRAS* (n=30; 4.6%) and *BRAF* (n=63; 9.6%). *KRAS* and *NRAS* mutations were mutually exclusive. *KRAS* and *NRAS* coexisted with *BRAF* mutations in four and in one instances, respectively. In most of these cases (4/5), *BRAF* mutations occurred outside of codon 600. *PIK3CA* gene mutations occurred in 98 (15%) cases. More frequently, *PIK3CA* mutations were detected together with other gene mutations; *PIK3CA* was the only mutated gene in 15/98 (15.3%) samples.

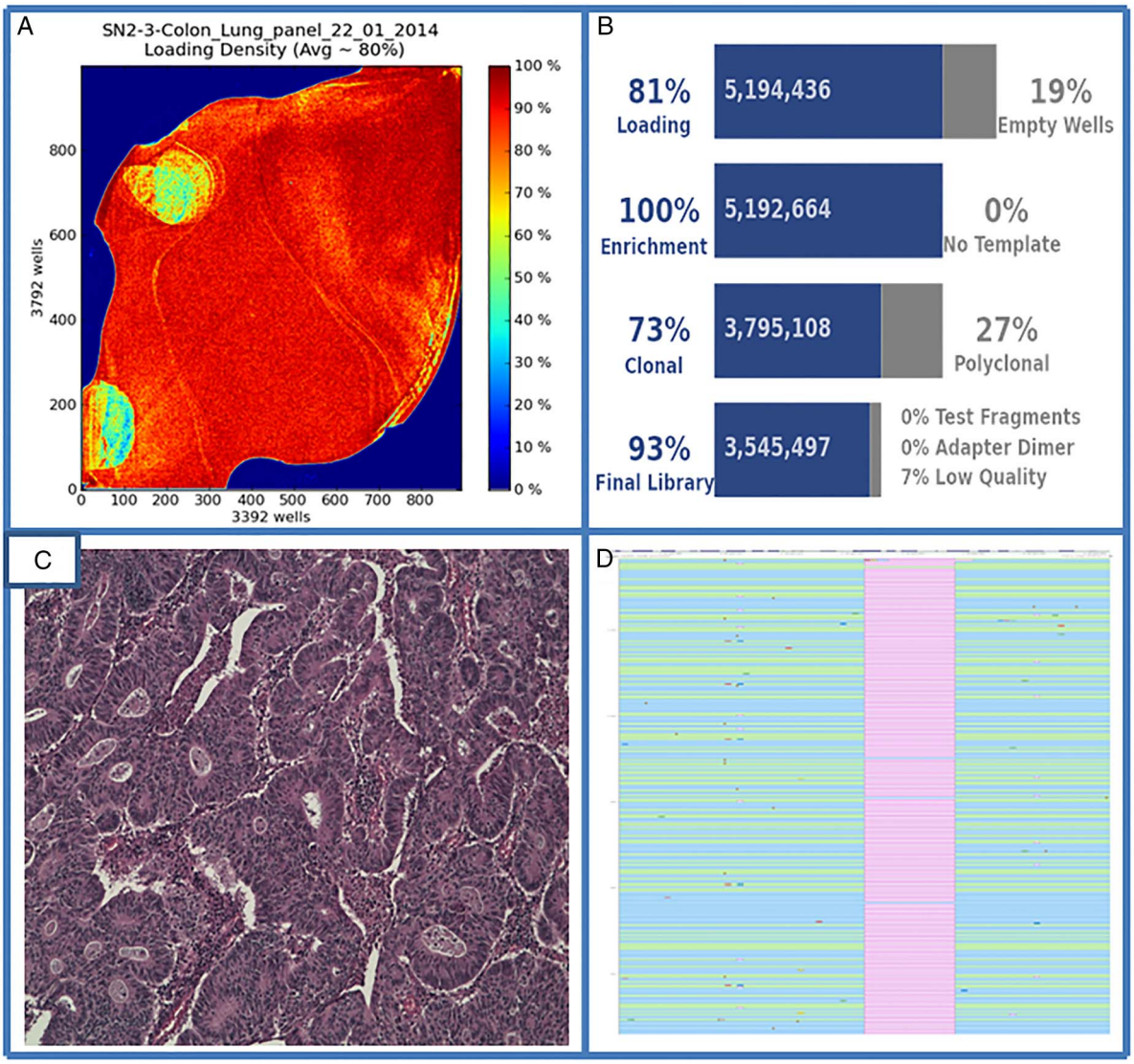

Figure 1 Loading density (A) and performance parameters (B) of an Ion Torrent sequencing run, carried out using a 316 chip, are shown. DNA extracted from the colorectal cancer (CRC) shown in (C) harboured an epidermal growth factor receptor p.E746_A750delELREA mutation. (D) was observed with a Genome Brower web app.

Table 2 Number and percentage of cases of each gene sequenced by the Ion Torrent AmpliSeq Colon and Lung Cancer Panel

Gene	Number of mutated cases (%)
KRAS	247* (37.8%)
TP53	240† (36.8%)
PIK3CA	98‡ (15%)
BRAF	63 (9.6%)
FBXW7	39 (6%)
NRAS	30 (4.6%)
PTEN	18 (2.8%)
SMAD4	14 (2.1%)
EGFR	8 (1.2%)
CTNNB1	7 (1.1%)
AKT1	6 (0.9%)
STK11	5 (0.8%)
ERBB4	4 (0.6%)
ERBB2	4 (0.6%)
NOTCH1	1 (0.2%)
ALK	1 (0.2%)
MAP2K1	1 (0.2%)

Note: *DDR2*, *FGFR1*, *FGFR2*, *FGFR3* and *MET* genes did not harbour any alteration.
*4/247 cases harboured 2 *KRAS* mutations.
†5/240 cases harboured 2 *TP53* mutations.
‡1/98 cases harboured 2 *PIK3CA* mutations.

Number and percentage of mutated cases of each gene are reported in table 2 and exons and codons involved are detailed in online supplementary information (file 4).

Besides *RAS/RAF/PI3KA* and *TP53* gene mutations, the Ion AmpliSeq Colon and Lung Cancer Panel provided information on additional targets, such as RTK genes, RTK signalling genes and other well-known cancer-related genes, as it follows.

RTK gene mutations

ALK: in one case (0.2%) the p.L1196M mutation was detected in association with two mutations of the *TP53* gene. ***EGFR***: mutations occurred in eight (1.2%) cases, with exon 19 deletion evident in four instances (n=3 p.E746_E749delELRE; n=1 p.E746_A750delELREA, as shown in figure 1). Most cases (7/8) were associated with other gene alterations; in particular, five cases harboured a KRAS mutation. ***ERBB2***: mutations occurred in four (0.6%) cases, with the V842I being detected in three instances. ***ERBB4***: mutations occurred in four cases (0.6%).

RTK signalling genes mutations

AKT1: the E17K mutation occurred in six cases (0.9%). ***PTEN***: mutations occurred in 18 (2.8%) cases. ***MAP2K1***: in one case (0.2%) the K57N mutation was associated with *PIK3CA* mutation. ***STK11***: mutations occurred in five cases (0.8%).

Other cancer-related genes

NOTCH1: mutation occurred in one case (0.2%) and remarkably this case had five additional gene mutations occurring in *TP53*, *KRAS*, *PTEN*, *ERBB4* and *PIK3CA*. ***CTNNB1***: mutations were detected in seven cases (1.1%), being always associated with at least one other concurrent mutation. In particular, *CTNNB1* mutations were consistently associated with the constitutive activation of the *RAF/MEK/ERK* pathway by either *KRAS* (n=4) or *BRAF* (n=3) concurrent mutations. ***SMAD4***: mutations were found in 14/653 (2.1%) samples, and in combination with other mutations (9/14). ***FBXW7***: mutations were identified in 39/653 patients (6%), singly (n=7) and associated with KRAS (n=20).

DISCUSSION

This study evaluated in CRC routine samples a broad set of genes for mutational events. Previous evidences regarding the *RAS/RAF/PI3KA* gene were confirmed. *KRAS* and *NRAS* mutations were always mutually exclusive,[5] whereas occasionally *BRAF* (mostly no V600E) mutations coexisted with an *RAS* gene alteration.[7] The frequent association of *PIK3CA* mutations with the *RAS/RAF* alterations was also confirmed.[5] Our data straighten the view that the simple distinction of tumours in *RAS*, *BRAF* or *PIK3CA* does not apply to CRC with combined *RAS/RAF* genetic changes.[7] We also confirmed that one of the most frequently mutated genes in CRC is *TP53*, whose mutation rate in our study was 38.8%.

Additional information was generated on other potentially actionable components of the CRC genomic landscape, such as RTK genes. Remarkably, the *ALK* p.L1196M gatekeeper mutation, which confers high-level resistance to crizotinib in lung cancer, was for the first time detected in CRC. *EGFR* mutations were also detected, as shown in figure 1, and their mutation rate (1.2%) was lower than that (4.5%) reported in the Tumor Cancer Genome Atlas (TCGA).[5] While *KRAS* and *EGFR* mutations are normally exclusive, concomitant *KRAS* and *EGFR* mutations were also detected (see online supplementary table S1), confirming previous NGS findings.[8] Other mutations include those involving *ERBB2*; in particular, the V842I *ERBB2* mutation associated with breast cancer[9] was detected in three instances. Remarkably, in CRC preclinical models *HER2* mutations were resistant to cetuximab and panitumumab and responsive to second-generation *HER2/EGFR* irreversible tyrosine, afatinib and neratinib.[10] Clinical trials targeting *HER2* activating mutations in metastatic CRC are ongoing.[11] *ERBB4* mutations occurring in 0.6% of the cases have an uncertain prognostic significance. In fact, the TCGA data set indicated a survival disadvantage in colorectal carcinoma with *ERBB4*,[5 12] whereas another study showed that the *ERBB4* mutant clones are not selected in metastatic spread.[13]

A number of rare mutations occurring in the *PI3K/AKT/mTOR* pathway are potentially actionable. As an example, *AKT1* mutations were associated with primary resistance to anti-*EGFR* therapy.[14] In our study, *AKT1* was mutated in 0.9% of cases, being mutually exclusive with *PIK3CA* alterations, as previously shown.[14] The recent association between E17K *AKT1* and tumours with mucinous morphology was observed only in one of our six cases.[14] Previous studies showed a wide range of *PTEN* mutation rates (0.7%[15] to 6%[16]). In our study, the mutation rate of *PTEN* was 2.8%. Interestingly, a total of 11 different mutations were found, according to the notion that mutations in tumour suppressor genes do not strongly cluster in single mutational hot spot.[17] Another RTK signalling gene included in our panel is the *STK11* gene. We confirm that somatic *STK11* mutations rarely occur in somatic CRC (0.8%).[18] Earlier studies reported that *STK11* mutant neoplasms had alterations in nucleotide metabolism that confer hypersensitivity to deoxythymidylate kinase inhibition, proposing that deoxythymidylate kinase is a possible therapeutic target.[19]

Interestingly, *CTNNB1* mutations detected in 1.1% of the cases were always associated with at least one other concurrent mutation (see online supplementary table S1). In particular, *CTNNB1* mutations were consistently associated with the constitutive activation of the *RAF/MEK/ERK* pathway by

either *KRAS* (n=4) or *BRAF* (n=3) concurrent mutations, in keeping with the notion that *CTNNB1* mutations are early events in CRC carcinogenesis.[20] Conversely, our data confirm that the occurrence of *SMAD4* mutations (2.1%) is a late event.[21] In fact, in our study 64.3% of *SMAD4* mutations occurred in combination with other alterations. *SMAD4* loss of function was associated with a worse prognosis and decreased disease-free survival and with resistance to 5-fluorouracil chemotherapy.[22 23] In this present study, *FBXW7*, a major tumour suppressor gene crucial in promoting exit from the cell cycle, was mutated in 6% of cases, which is in line with the estimated 9% of CRCs containing *FBXW7* mutations.[24 25] Preclinical data have suggested that inactivating mutations of *FBXW7* could predict sensitivity either to the *mTOR* inhibitor rapamycin,[26] or to the histone deacetylase inhibitor MS-275.[27] Noteworthy, as it was shown in previous reports *FBXW7* were often (51.2%) associated with *KRAS* mutations.[28 29] Interestingly, concurrent molecular aberrations can contribute to limited therapeutic efficacy of *mTOR* inhibitors in the presence of *FBXW7* mutations.

Certain genes included in our panel, such as *MAP2K1*, may have a future role in sensitivity, resistance or both, to a variety of preclinical drugs. Targeting of *NOTCH* signalling may be of therapeutic value in colon cancers, as activating mutations in *NOTCH-1* have been previously reported in colon cancer.[30] In our study *NOTCH* mutation occurred in one case (0.2%) and remarkably this case had five additional gene mutations occurring in *TP53*, *KRAS*, *PTEN*, *ERBB4* and *PIK3CA*.

In conclusion, our data confirm that CRCs consist of a group of heterogeneous disorders with a large number of diverse sets of genetic changes in oncogenes and tumour suppressor genes. In a routine diagnostic setting, the Ion PGM and AmpliSeq colon and Lung Cancer Panel had the potential to exploit even a low-input DNA to uncover multiple common mutations simultaneously and to generate robust and comprehensive genetic information. Several updates of the Ion Torrent system may soon enable to detect also gene copy number alterations and translocations to more comprehensively cover the whole spectrum of genomic alterations refining the identification of reliable and reproducible biomarkers of response/resistance to the targeted treatment of CRC.

Take home messages

- Ion Torrent Personal Genome Machine (PGM), and the Ion AmpliSeq Colon and Lung Cancer Panel, enabled our laboratory to adopt next-generation sequencing.
- Less information is available on the uncommon mutated genes of the CRC genomic landscape.
- In a routine diagnostic setting, the AmpliSeq Colon and Lung Cancer Panel had the potential to generate robust and comprehensive genetic information.

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Contributors UM, PP and GT conceived the study and wrote the paper. RS performed the experimental part. EV, GG and CB contributed as pathologists. CC and MB contributed as oncologists.

Competing interests None declared.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Malapelle U, Carlomagno C, de Luca C, *et al*. KRAS testing in metastatic colorectal carcinoma: challenges, controversies, breakthroughs and beyond. *J Clin Pathol* 2014;67:1–9.
2 Douillard JY, Siena S, Cassidy J, *et al*. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010;28:4697–705.
3 Tops BB, Normanno N, Kurth H, *et al*. Development of a semi-conductor sequencing-based panel for genotyping of colon and lung cancer by the Onconetwork consortium. *BMC Cancer* 2015;15:26.
4 Malapelle U, Vigliar E, Sgariglia R, *et al*. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. *J Clin Pathol* 2015;68:64–8.
5 Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330–7.
6 Wong NA, Gonzalez D, Salto-Tellez M, *et al*. RAS testing of colorectal carcinoma-a guidance document from the Association of Clinical Pathologists Molecular Pathology and Diagnostics Group. *J Clin Pathol* 2014;67:751–7.
7 Normanno N, Rachiglio AM, Lambiase M, *et al*. Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. *Ann Oncol* 2015;26:1710–4.
8 Chevrier S, Arnould L, Ghiringhelli F, *et al*. Next-generation sequencing analysis of lung and colon carcinomas reveals a variety of genetic alterations. *Int J Oncol* 2014;45:1167–74.
9 Weigelt B, Reis-Filho JS. Activating mutations in HER2: neu opportunities and neu challenges. *Cancer Discov* 2013;3:145–7.
10 Greulich H, Kaplan B, Mertins P, *et al*. Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2. *Proc Natl Acad Sci USA* 2012;109:14476–81.
11 Kavuri SM, Jain N, Galimi F, *et al*. HER2 activating mutations are targets for colorectal cancer treatment. *Cancer Discov* 2015;5:832–41.
12 Williams CS, Bernard JK, Demory Beckler M, *et al*. ERBB4 is over-expressed in human colon cancer and enhances cellular transformation. *Carcinogenesis* 2015;36:710–18.
13 Kogita A, Yoshioka Y, Sakai K, *et al*. Inter- and intra-tumor profiling of multi-regional colon cancer and metastasis. *Biochem Biophys Res Commun* 2015;458:52–6.
14 Hechtman JF, Sadowska J, Huse JT, *et al*. AKT1 E17K in colorectal carcinoma is associated with BRAF V600E but not MSI-H status: a clinicopathologic comparison to PIK3CA helical and kinase domain mutants. *Mol Cancer Res* 2015;13:1003–8.
15 Lan YT, Jen-Kou L, Lin CH, *et al*. Mutations in the RAS and PI3K pathways are associated with metastatic location in colorectal cancers. *J Surg Oncol* 2015;111:905–10.
16 Day FL, Jorissen RN, Lipton L, *et al*. PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer. *Clin Cancer Res* 2013;19:3285–96.
17 Stachler MD, Rinehart E, Lindeman N, *et al*. Novel molecular insights from routine genotyping of colorectal carcinomas. *Hum Pathol* 2015;46:507–13.
18 Avizienyte E, Roth S, Loukola A, *et al*. Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. *Cancer Res* 1998;58:2087–90.
19 Liu Y, Marks K, Cowley GS, *et al*. Metabolic and functional genomic studies identify deoxythymidylate kinase as a target in LKB1-mutant lung cancer. *Cancer Discov* 2013;3:870–9.
20 Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol* 2011;6:479–507.
21 Fleming NI, Jorissen RN, Mouradov D, *et al*. SMAD2, SMAD3 and SMAD4 mutations in colorectal cancer. *Cancer Res* 2013;73:725–35.
22 Zhang B, Zhang B, Chen X, *et al*. Loss of Smad4 in colorectal cancer induces resistance to 5-fluorouracil through activating Akt pathway. *Br J Cancer* 2014;110:946–57.
23 Alhopuro P, Alazzouzi H, Sammalkorpi H, *et al*. SMAD4 levels and response to 5-fluorouracil in colorectal cancer. *Clin Cancer Res* 2005;11:6311–6.
24 Akhoondi S, Sun D, von der Lehr N, *et al*. FBXW7/hCDC4 is a general tumor suppressor in human cancer. *Cancer Res* 2007;67:9006–12.
25 Bamford S, Dawson E, Forbes S, *et al*. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* 2004;91:355–8.
26 Wang Y, Liu Y, Lu J, *et al*. Rapamycin inhibits FBXW7 loss-induced epithelial-mesenchymal transition and cancer stem cell-like characteristics in colorectal cancer cells. *Biochem Biophys Res Commun* 2013;434:356–6.

27 Yokobori T, Yokoyama Y, Mogi A, *et al*. FBXW7 mediates chemotherapeutic sensitivity and prognosis in NSCLCs. *Mol Cancer Res* 2014;12:32–7.
28 Jardim DL, Wheler JJ, Hess K, *et al*. FBXW7 mutations in patients with advanced cancers: clinical and molecular characteristics and outcomes with mTOR inhibitors. *PLoS ONE* 2014;9:e89388.
29 Bai J, Gao J, Mao Z, *et al*. Genetic mutations in human rectal cancers detected by targeted sequencing. *J Hum Genet* 2015;60:589–96.
30 Fender AW, Nutter JM, Fitzgerald TL, *et al*. Notch-1 promotes stemness and epithelial to mesenchymal transition in colorectal cancer. *J Cell Biochem* 2015;116:2517–27.

Introduzione. L'incidenza delle mutazioni nei geni RAS/RAF/PI3KA e TP53 sono ben stabilite nel carcinoma del colon-retto (CRC). Invece, relativamente alle altre componenti del panorama genomico del CRC, che potrebbero essere potenziali marcatori prognostici/predittivi, sono disponibili minori informazioni. **Metodi**. In seguito ad uno studio precedente di validazione, la piattaforma *Personal Genome Machine* (PGM) di sequenziamento di nuova generazione (NGS) è stata poi impiegata per processare 653 campioni di routine del CRC impiegando un pannello di 22 geni significativi per CRC. **Risultati.** Sono state rilevate 796 mutazioni somatiche in 499 (76.4%) tumori. Insieme a RAS/RAF/PI3KA e TP53, altri 12 geni hanno mostrato almeno una mutazione, tra questi FBXW7 (6%), PTEN (2.8%), SMAD4 (2.1%), EGFR (1.2%), CTNNB1 (1.1%), AKT1 (0.9%), STK11 (0.8%), ERBB2 (0.6%), ERBB4 (0.6%), ALK (0.2%), MAP2K1 (0.2%) e NOTCH1 (0.2%). **Conclusioni.** Nella pratica diagnostica routinaria, il sequenziamento genico di nuova generazione ha il potenziale di generare molte informazioni e robuste anche riguardo mutazioni geniche meno frequenti ma potenzialmente rilevanti come marcatori prognostici e predittivi di risposta al trattamento.

Methods for molecular profiling of tumor samples by next generation sequencing.

Protocol and Ethical issues.

Our molecular laboratory is an accredited Italian Society of Pathology reference centre for RAS testing and the organiser in Italy for the ESP Colon External Quality Assessment Scheme. After obtaining the patient's consent, oncologists and the primary pathologists from outside institutions record the clinical and pathological data (including the original pathology report) on a dedicated website. Then, the corresponding tissue sample is express-mailed to our central laboratory. Upon receipt of each sample, a representative H&E stained slide is reviewed by a pathologist and the area with the highest density of neoplastic cells is marked, annotating the percentage of neoplastic cells.

Since RAS mutational analysis is the standard of care in diagnostic workup of patients with CRC, and our analysis did not interfere anyhow with the patient management, the need for ethic committee's approval was not necessary for this study, in accordance with medical ethical guidelines of the Università degli Studi di Napoli Federico II and in accordance with general authorisation to process personal data for scientific research purposes from 'The Italian Data Protection Authority', All samples and clinical data used in this study have been irreversibly anonymized.

Depending on the complexity of histology and on the density of the tumour, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) from two (resection specimens) or three (biopsy specimens) 10 µm-thick serial sections. An additional section (biopsy specimens only) was stained by H&E to confirm tumour cell percentage. DNA was extracted from cell lines and clinical tissue samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was suspended in 30 µL of molecular biology water. DNA quantity and quality were assessed using the Qubit photometer (Life Technologies) and the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions.

According to the manufacturer's protocols, 10 ng of DNA for each sample was used for library preparation with the Ion AmpliSeq Library 96LV Kit 2.0 (Life Technologies) and the Colon and Lung Cancer Panel (Life Technologies). This panel gives 90 amplicons covering 504 mutational hotspot regions in 22 genes (AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, TP53), with performance of at least 500× sequence coverage for eight samples on one Ion 316 chip. For samples yielding less than 10 ng DNA input, additional cycling conditions were used for library preparation as recommended by

the manufacturer. Each library was barcoded with the Ion Xpress Barcode Adapters 1–16 Kit (Life Technologies). Barcoded libraries were combined to a final concentration of 100 pM. Template preparation by emulsion PCR (emPCR) was performed on the Ion OneTouch 2 system (Life Technologies). Library quality control was performed using the Ion Sphere Quality Control Kit according to the manufacturer's instructions, ensuring that 10–30% of template positive Ion Sphere particles (ISP) were targeted in the emPCR reaction. Sequencing primer and polymerase were added to the final enriched ISPs prior to loading onto 316 (100 Mb output) chips. Sequencing was carried out on the PGM (Life Technologies). Data analysis was carried out with Torrent Suite Software V.3.2 (Life Technologies). After alignment to the hg19 human reference genome, the Variant Caller plug-in was applied using the Colon and Lung hotspot file as a reference (downloaded from Ion Community, http://www.ioncommunity.lifetechnologies.com, last accessed 1 September 2015). The Ion Reporter suite (Life Technologies) was used to filter polymorphic variants. In addition, all nucleotide variations with less than a 5% variant frequency were masked. All detected variants were manually reviewed with the Integrative Genomics Viewer (IGV V.2.1, Broad Institute, Cambridge, Massachusetts, USA) or with Genome Brower web app.

Performance parameters

In all cases analyzed, a 100 pM DNA library was obtained; only in 24 cases, the library preparation procedure was repeated, after an initial failure. While most cases yielded a DNA input > 10 ng, eight samples did not satisfied this request. However, even for these cases an increase in the number of amplification cycles enabled to get an adequate library. An average of 3.9 million of the total 6.3 million addressable wells in the Ion 316 chip were consistently loaded with ISPs, and 3.2 million (92%) of these particles contained library templates. After subtraction of multiple-templated beads and poor quality sequence reads, an average of 2.7 million reads were obtained. Samples averaged 193,000 mapped sequence reads (range, 10,331 to 1,010,971) with a mean read length was 115 bp. Multiplex PCR mediated target capture was very effective, as an average of 93.5% of the sequence reads mapped to targeted gene regions. The distribution of reads across the 90 amplicons was consistent across samples and there was an average of 1930 reads per amplicon (range, 102 to 10982).

patient	cr 1 DDR2	cr 1 NRAS	cr 2 ALK	cr 2 ERBB4	cr 3 CTNNB1
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cr 3	cr 4	cr 4	cr 7	cr 7
PIK3CA	FBXW7	FGFR3	BRAF	EGFR
E542K				
			G466E	
E542K				
H1047R				
			D594G	
	R385C			
E542K				
	R278*			
			V600E	
H1047R	R385C			
Q546P				
	R465H			

H1047R

R385C V600E

R465H

E545K G469E

V600E

S582L

H1047R

E545K

delELRE

polyT ex20

V600E

E542Q

V600E

R465H

E542K

V600E

R465H

E545K

H1047L

Q546K

V600E

E545K
E542K

G466E

delELRE

E545K

H1047R

R776H

R278*

R385C

V600E

E542K
E542K

V600E

E542K

N581S

E545K

E545K

E545K

E545K

R385C

M1040I

Q546H
E542K

E545K

T599I

V600E

V600E

E542K

V600E

Q546R

E545K

V600E

E545K R465H

E542K

E545K

R385C

V600E

V600E

E542K + H1047Y V600E

H1047R

E545K

E542K

E542K V600E

D594N

E545Q

R465H

Q546K

H1047R V600E

R465H

V600E

G469R

R385C V600E

E545K

R266C

V600E

R278*

Q546K

E545K

E542K

E545K

V600E

Q546K R266C

R266C V600E

E545K

E545K

H1047L

E545K

H1047R

H1047R

V600E

V600E

E545K

V600E

E545K

R465H

H1047R R776C

E542K

E542K V600E

R505L

H1047R

delELREA

M1043I

H1047R
H1047R

T1025A

Q546K

N581S
R266C

H1047L

E545K

E542K

R266C

G466V

E545K R465H

E545K R266C

R385C

E542K
E542K

R385C V600E

E542K L597R
E542K L597R

H1047R

E545K

N581S

H1047R		V600E	
H1047R			
E545K			
	R266C	G469A	
		V600E	
	S582L		
E545K			
polyT ex20	R278*		
T1025A		V600E	
			S784F
			G874S
		V600E	

V600E

E542K

V600E

E545K

G1049R

E542K R465H

V600E

R266C

		V600E
		V600E
polyT ex20		
E545K		
	R385C	V600E
E542K	R385C	V600E
	R266C	delELRE
		V600E
		V600E
E542K		
E545K		V600E

H1047Y	R465H	
		V600E
		V600E
		N581S
H1047R		
		N581S
		G466V
E545K		
Y1021C		
		V600E
	R278*	G466V

cr 7	cr 8	cr 9	cr 10	cr 10	cr 12	cr 14	cr 15
MET	FGFR1	NOTCH1	FGFR2	PTEN	KRAS	AKT1	MAP2K1
					G13D		
					G12V		
					G12D		
					G12D		
					G12D		
				E242fs*	G12D		
					G12D		
					G12C		
					Q61R		
					G12V		
					A146T		
					G12D		
					G12A		
					G12V		
					G12A		
					G12V		
					A146T		
					G13C		

	G13D
	A146T
	G12C
	G12D
	G12V
	G13D + G12C
	G12V
	G12D
	G13D
	G12C
p.?	G12V
	G12D
	G12D
	G13D
	G12V
	G12D
	G12C
	G12V

G12D
Q61H

G12D
A59T

G12V
G13D

Q61H

G12V

G12D

G13D
G12D

Q22K

G12C

G12V
G12R

G12D

G12A
G12V

E17K

	G12D
	G12D
	A146T
	G12V
	G12D
	G12D
	G13D
	G12D
	G12D
	K117N
	Q61L
L57fs*	
D252Y	A146T
	G12V
	G12V
	G12D
	G12D
	G13D
T321fs*	
	G12D
	G12A
	G12C
	G13D
p.?	
	G12S
	G12S
	A146T
	Q61H

	G12S
	K117N
	G12D
	G12D
	G12A
	A146V
	G12V
	G12D
	K117N
	G12C
	G12D
G165E	G12S
	G12S
T321fs*	
	G12V
	G12D
	G12D
	G12D
	G13D
	G12D
	G12F

G13D

G12A
G13D

G13C

G12S + A11V
G12S

G13D
G12D
G13C

G12V
G13D
G12V
G12D
G12D

Q61H

I253N
G12D

G13D
G13D

G12V

G13D E17K
G12V
G12V

G12C

A146T E17K

G12V
G13D
G12V
P339S

G12C

G12V
G12V
G12V
G12D

G13D E17K

Q61L

A146T
G12D

G13insG

G12D
G12D
G12C

G12V
G12D
G12C

G12V
G12V
Q61K
G12V

		G12D
		G12V
		G12A
	R173H	A146T
		G12V
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		G13D
		G12V
		G12V
V1578delV	D252Y	G12V
		Q61H
		G13D
		G12V
		G12V
		G12A

A146P
Q61L
G12V

G12V

G12V

L318fs*
G12S
G12D

G13D
G12V
G12V

G12A
G12D

G12D
G13D
K57N
A146P

G12V
L19F
G13D
G13D

G12D
G12V

G13D

G12C
G12V

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G12V
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Q61L
A146T

K117N
G12D
G13D
G12C

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G13D

G12C

G12D

A146T

p.?

G12S
G12V

A146T

A146T

E242*

A146T
G13D + G12V

G13D + G12D

E17K

G12S

A59E

Q61H

G12V

L318fs*

K117N

Q61H

A59T

G12V

G12V

K117N

G12V

G12A

G12D
Q61H

G12D
A146T

A146V

G12S

G13D

S170N

G12V

E17K

K117N

Q61L

R173H Q61L

A146T

K117N

L19F

cr 17	cr 17	cr 18	cr 19
ERBB2	TP53	SMAD4	STK11
	R156fs*		
	E286fs*		P281fs*
	Y205H		
			P281fs*
	Y234N		
	R175H		
	E198K		
	R306*		
	R342*		
	A276D		
	R175H		
	R283C		
	R175H		
	K132R		
	P278fs*		
	Q104*		
	R213fs*		

R65H

Y234H
E204*
Q104*

Y220C
R196*
N247T
R306*
V274A
P250L

V272L
R196*

M246K
N200fs*

P190T

R361H

R175H

H179N

E171*

S166*
R306*

R306*
R196*

G199V

C238R

N200fs*
P152S

E285K

R306*

I195T

R175H
R306*

E285K
A118V
K132N
W91*

S241fs*
R175H

V80M

S166*
R342*

R306*

L265R
p.?
E271K + R175H

S215R
R196*

R306*

	R175H		
	R175H		
		R361H	
	R175H		
	R306*		
	R175H		
	p.?		
	C135F		
	R175H		
		R361H	
	R175H		
V842I	R175H	R361H	
	R342*		
	P219S		
	I255S		
	G105fs*		
		R361H	
			P281fs*
	R175H		
	R306*		
	Y220C		
	E298*		

R175H

E204*
F212fs*
R175H
R175H

R196*

R175H

R280K
R175H
R65H

G245V
Y220C

P190fs*

G187S
G266E
Y236C
G266E
R175H

P152L
E204*

E171*

E294fs*

R213*

R196*

P152L

p.? A118V

R175H

P278R + F270V

C242F

R196*

R342*

H179Q

R65H

R342* + P295S

C238Y

N239S
R209fs*

C229fs*

R175H

K132R

R175H

R175H
I251fs*
I254S

R283H

C238Y

G168*

I195T

R196*
E298* + V80M

R175H
R196*

R361H

R175H

K132Q

A118V

R175H

R342*

E271K
R65H

R175H
H193Y

C242fs*
C242fs*
p.?
P278R

I255S
R213*

L282fs*

G245V
S183*

R65H
R175H

S241Y

R175H

V80M

I195S
R213*
R306*

R361H

R175H

R361H

R175H

C176F

R175H

p.?

S94*

T231I

V842I

R175H

I232S

R175H

R280G
R175H

R110C
I195T
R267W

E204*
N288fs*
R156fs*

S215G

Y220C
R175H

p.?
Y236N
R175H
R175H
P281fs*

R306*
C135F
R361H

R175H

R213*
R306*

R175H

H168R

R342*

R249S

T253P

G776V

R175H

R175H

R342*
R342*
R213*

R213*
R175H

R267W

C242fs*

R175H

R175H

V272L

C275F

E336*
G187S

R175H
R196*

R213*
R175H
Y220C

E271K

R213*

R175H

C135F

R213*
E285K

R306* + R283C

R196*
R196*

R175H
R283C

P177R
p.?

Q245*

I195T

R306*

V842I

p.?
R175H
R175H

R65H

R213*

P190L

C176F

R306*

N131delN

V80M
R249M
A159D
R306*

V274F

P152L

Patient	cr 1 DDR2	cr 1 NRAS	cr 2 ALK	cr 2 ERBB4	cr 3 CTNNB1
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cr 3	cr 4	cr 4	cr 7	cr 7
PIK3CA	FBXW7	FGFR3	BRAF	EGFR

F384L

F384L

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F384L

cr 7	cr 8	cr 9	cr 10	cr 10	cr 12	cr 14	cr 15
MET	FGFR1	NOTCH1	FGFR2	PTEN	KRAS	AKT1	MAP2K1

N375S

N375S

N375S

T1010I

R173C

N375S

T1010I

E168D

T1010I
N375S

N375S

N375S

N375S

N375S

N375S

T1010I

N375S

N375S

N375S

N375S

N375S

R173C

cr 17	cr 17	cr 18	cr 19
ERBB2	TP53	SMAD4	STK11
	R282W		
	G245S		
	R273H + R213Q		
	R248W		
	R273C		
	R248Q R282W		
	R273C		

R282W

R273H

F354L

R248W
Y236D

R361C

R248W

V272M
R248Q
R273H

R248W

G245D

R273C

R273C

R273C

G245C

R273H

Y163C

R273H

R361C

R282W

R282W

R248W
C176Y

R273C

G245S

R248Q R361C

R267Q

R248Q
R248Q

F354L

R248W
R248W
R282W

R273C

R248Q

G245S

G245S
V272M
V272M

G245D
R273C

R282W

R273C

G266R
G245S

R361C

G245S

R248W

R248Q

R248W
M237I

V272M

P281L

R248Q
R273H

R273H R361C

C275Y

R361C
R337C
R361C
H193L

R337C R361C

R273H
R248Q

D208V

R273H

G245D R361C

Y126D

V216M

R282W
R248W

S127F + S99F
R273C

G245S
G244D
R273H

R273C

R273H

R273H

R273H
R248W

R282W

R249K
G244D

R248Q

R282W

F354L

R337C

R273H

R273H

R282W

G245S

R273H

G245S
R273H

R273C R361C

R273C

R248Q

R282W
R273H
R273C

R273C

R213Q

M237I

G245S
R213Q

R248Q
E285K

R248Q
G244D

R273C
R273C

R282W

R282W

R273C

R248Q F354L

R282W

R248Q

R273H
R273H

G245S
R273H

G245S
V216M

R337L

R248W

R282W
R248Q
R181C

Y163C

F354L

R282G

F354L

G245S

D281G

R248Q

R248W

R273C

R248Q

G244D

R248W

R248W

R273H

R248W

M237I

G245S

R273C
R282W

V216M

G245S

G245S
M237V

Y236H
R248W

E286K

G245D

R248Q

KRAS

Total: 251

Exon 2: 204/251 (A11V; G12A/C/D/F/R/S/V; G13C/D; G13insG; L19F; Q22K)

Exon 3: 19/251 (A59E/T; Q61H/K/L/R)

Exon 4: 28/251 (K117N; A146P/T/V)

NRAS

Total: 30

Exon 2: 12/30 (G12C/D/V; G13R/V)

Exon 3: 18/30 (Q61H/K/L/R)

BRAF

Total: 63

Exon 11: 8/63 (G466E/V; G469A/E/R)

Exon 15: 55/63 (N581S; D594G/N; L597R; T599I; V600E)

PIK3CA

Total: 99

Exon 9: 68/99 (E542K/Q; E545K/Q; Q546H/K/P/R)

Exon 20: 31/99 (Y1021C; T1025A; M1040I; M1043I; H1047L/R/Y; G1049R; polyT ex20)

TP53

Total: 245

Exon 4: 16/245 (R65H; V80M; W91*; S94*; Q104*; G105fs*; R110C)

Exon 5: 83/245 (N131delN; K132N/Q/R; C135F; P152L/S; R156fs*; A159D; S166*; H168R; E171*; R175H; C176F; P177R; H179N/Q; S183*; p.?)

Exon 6: 57/245 (G187S; p190fs*; p190L/T; H193Y; I195S/T; R196*; E198K; G199V; N200fs*; E204*; Y205H; R209fs*; F212fs*; R213fs*; S215G/R; P219S; Y220C; p.?)

Exon 7: 29/245 (C229fs*; T231I; I232S; Y234H/N; Y236C/N; C238R/Y; N239S; S241fs*; S241Y; C242F; C242fs*; G245V; M246K; N247T; R249M/S; P250L; I251fs*; T253P; I254S; I255S)

Exon 8: 50/245 (L265R; G266E; R267W; F270V; E271K; V272L; V274A/F; C275F; A276D; P278fs*; P278R; R280G/K; R283C/H; E285K; E286fs*; N288fs*; E294fs*; P295S; E298*; R306*)

Exon 10: 10/245 (E336*; R342*)

SUPPLEMENTARY TABLE 1

Co-existing mutations in different genes

Multiple gene mutations in KRAS mutated tumors	
Type of mutations	n
KRAS only (no other mutation detected)	99
KRAS + TP53	62
KRAS + PIK3CA	31
KRAS + PTEN	4
KRAS + FBXW7	4
KRAS + STK11	2
KRAS + AKT1	2
KRAS + SMAD4	2
KRAS + EGFR	2
KRAS + BRAF	1
KRAS + ERBB2	1
KRAS + CTNNB1	1
KRAS + PIK3CA + TP53	10
KRAS + FBXW7 + TP53	6
KRAS + PIK3CA +FBXW7	3
KRAS + PIK3CA + BRAF	1
KRAS + PIK3CA +EGFR	1
KRAS + PIK3CA + PTEN	1
KRAS + PIK3CA + ERBB2	1
KRAS + FBXW7 + EGFR	1
KRAS + FBXW7 + AKT1	1
KRAS + BRAF + TP53	1
KRAS + PTEN + TP53	1
KRAS + TP53 + SMAD4	1
KRAS + CTNNB1 + PIK3CA + FBXW7	2
KRAS + CTNNB1 + PIK3CA + TP53	1
KRAS + PIK3CA + FBXW7 +TP53	1
KRAS + BRAF + FBXW7 + TP53	1
KRAS + ERBB4 + PIK3CA + FBXW7 + TP53	1
KRAS + ERBB4 + PIK3CA + EGFR + TP53	1
KRAS + + ERBB4 + PIK3CA + NOTCH1 + PTEN + TP53	1
Total KRAS mutated cases	247
4 tumor had two concomitant KRAS mutations	

Multiple gene mutations in TP53 mutated tumors	
Type of mutations	n
TP53 only (no other mutation detected)	103
TP53 + KRAS	62
TP53 + NRAS	13
TP53 + PIK3CA	10
TP53 + BRAF	10
TP53 + FBXW7	3

TP53 + PTEN	2
TP53 + EGFR	1
TP53 + ALK	1
TP53 + STK11	1
TP53 + PIK3CA + KRAS	10
TP53 + FBXW7 + KRAS	6
TP53 + PIK3CA + NRAS	1
TP53 + NRAS + PTEN	1
TP53 + CTNNB1 + BRAF	1
TP53 + BRAF + KRAS	1
TP53 + BRAF + PTEN	1
TP53 + EGFR + AKT1	1
TP53 + PTEN + KRAS	1
TP53 + PTEN + SMAD4	1
TP53 + KRAS + SMAD4	1
TP53 + ERBB2 + SMAD4	1
TP53 + PIK3CA + FBXW7 + KRAS	1
TP53 + NRAS +PIK3CA + BRAF	1
TP53 + PIK3CA + CTNNB1 + KRAS	1
TP53 + FBXW7 + BRAF + SMAD4	1
TP53 + FBXW7 + BRAF + KRAS	1
TP53 + ERBB4 + PIK3CA + FBXW7 + KRAS	1
TP53 + ERBB4 + PIK3CA + EGFR + KRAS	1
TP53 + ERBB4 + PIK3CA + NOTCH1 + PTEN + KRAS	1
Total TP53 mutated cases	240
5 tumor had two concomitant TP53 mutations	

Multiple gene mutations in PIK3CA mutated tumors	
Type of mutations	n
PIK3CA only (no other mutation detected)	15
PIK3CA + KRAS	31
PIK3CA + TP53	10
PIK3CA + BRAF	7
PIK3CA + NRAS	3
PIK3CA + FBXW7	2
PIK3CA + PTEN	1
PIK3CA + MAP2K1	1
PIK3CA + KRAS + TP53	10
PIK3CA + FBXW7 + KRAS	3
PIK3CA + BRAF + CTNNB1	1
PIK3CA + NRAS + PIK3CA	1
PIK3CA + FBXW7 + BRAF	1
PIK3CA + BRAF + KRAS	1
PIK3CA + EGFR + KRAS	1
PIK3CA + PTEN + KRAS	1
PIK3CA + KRAS + ERBB2	1
PIK3CA + CTNNB1 + FBXW7 + KRAS	2
PIK3CA + NRAS + BRAF + TP53	1

PIK3CA + CTNNB1 + KRAS + TP53	1
PIK3CA + FBXW7 + KRAS + TP53	1
PIK3CA + ERBB4 + FBXW7 + KRAS + TP53	1
PIK3CA + ERBB4 + EGFR + KRAS + TP53	1
PIK3CA + ERBB4 + NOTCH1 + PTEN +KRAS + TP53	1
Total PIK3CA mutated cases	98
1 tumor had two concomitant PIK3CA mutations	

Multiple gene mutations in BRAF mutated tumors	
Type of mutations	n
BRAF only (no other mutation detected)	26
BRAF + TP53	10
BRAF + PIK3CA	7
BRAF + FBXW7	5
BRAF + PTEN	2
BRAF + SMAD4	1
BRAF + KRAS	1
BRAF + CTNNB1	1
BRAF + PIK3CA + FBXW7	1
BRAF + CTNNB1 + TP53	1
BRAF + PIK3CA + CTNNB1	1
BRAF + KRAS + PIK3CA	1
BRAF + PTEN + SMAD4	1
BRAF + PTEN + TP53	1
BRAF + KRAS + TP53	1
BRAF + NRAS + PIK3CA + TP53	1
BRAF + FBXW7 + SMAD4 + TP53	1
BRAF + FBXW7 + KRAS + TP53	1
Total BRAF mutated cases	63

Multiple gene mutations in FBXW7 mutated tumors	
Type of mutations	n
FBXW7 only (no other mutation detected)	7
FBXW7 + BRAF	5
FBXW7 + KRAS	4
FBXW7 + TP53	3
FBXW7 + PIK3CA	2
FBXW7 + KRAS + TP53	6
FBXW7 + PIK3CA + KRAS	3
FBXW7 + PIK3CA + BRAF	1
FBXW7 + EGFR + KRAS	1
FBXW7 + KRAS + AKT1	1
FBXW7 + CTNNB1 + PIK3CA + KRAS	2
FBXW7 + PIK3CA + KRAS + TP53	1
FBXW7 + BRAF + TP53 + SMAD4	1

FBXW7 + KRAS + BRAF + TP53	1
FBXW7 + ERBB4 + PIK3CA + KRAS + TP53	1
Total FBXW7 mutated cases	39

Multiple gene mutations in NRAS mutated tumors	
Type of mutations	n
NRAS only (no other mutation detected)	11
NRAS + TP53	13
NRAS + PIK3CA	3
NRAS + PIK3CA + TP53	1
NRAS + PTEN + TP53	1
NRAS + PIK3CA + BRAF + TP53	1
Total NRAS mutated cases	30

Multiple gene mutations in PTEN mutated tumors	
Type of mutations	n
PTEN only (no other mutation detected)	2
PTEN + KRAS	4
PTEN + BRAF	2
PTEN + TP53	2
PTEN + PIK3CA	1
PTEN + KRAS + TP53	1
PTEN + BRAF + SMAD4	1
PTEN + PIK3CA + KRAS	1
PTEN + NRAS + TP53	1
PTEN + BRAF + TP53	1
PTEN + TP53 + SMAD4	1
PTEN + ERBB4 + PIK3CA + NOTCH1 + KRAS + TP53	1
Total PTEN mutated cases	18

Multiple gene mutations in SMAD4 mutated tumors	
Type of mutations	n
SMAD4 only (no other mutation detected)	5
SMAD4 + KRAS	2
SMAD4 + BRAF	1
SMAD4 + AKT1	1
SMAD4 + BRAF + PTEN	1
SMAD4 + ERBB2 + TP53	1
SMAD4 + PTEN + TP53	1
SMAD4 + KRAS + TP53	1
SMAD4 + FBXW7 + BRAF + TP53	1
Total SMAD4 mutated cases	14

Multiple gene mutations in EGFR mutated tumors	
Type of mutations	n
EGFR only (no other mutation detected)	1
EGFR + KRAS	2
EGFR + TP53	1
EGFR + PIK3CA + KRAS	1
EGFR + AKT1 + TP53	1
EGFR + KRAS + FBXW7	1
EGFR + ERBB4 + PIK3CA + KRAS + TP53	1
Total EGFR mutated cases	8

Multiple gene mutations in CTNNB1 mutated tumors	
Type of mutations	n
CTNNB1 only (no other mutation detected)	0
CTNNB1 + BRAF	1
CTNNB1 + KRAS	1
CTNNB1 + BRAF + TP53	1
CTNNB1 + PIK3CA + BRAF	1
CTNNB1 + PIK3CA + FBXW7 + KRAS	2
CTNNB1 + PIK3CA + KRAS + TP53	1
Total CTNNB1 mutated cases	7

Multiple gene mutations in AKT1 mutated tumors	
Type of mutations	n
AKT1 only (no other mutation detected)	1
AKT1 + KRAS	2
AKT1 + SMAD4	1
AKT1 + EGFR + TP53	1
AKT1 + FBXW7 + KRAS	1
Total AKT1 mutated cases	6

Multiple gene mutations in STK11 mutated tumors	
Type of mutations	n
STK11 only (no other mutation detected)	2
STK11 + KRAS	2
STK11 + TP53	1
Total STK11 mutated cases	5

Multiple gene mutations in ERBB4 mutated tumors	
Type of mutations	n
ERBB4 only (no other mutation detected)	1
ERBB4 + PIK3CA + FBXW7 + KRAS + TP53	1
ERBB4 + PIK3CA + EGFR + KRAS + TP53	1
ERBB4+ PIK3CA + NOTCH1 + PTEN + KRAS + TP53	1
Total ERBB4 mutated cases	4

Multiple gene mutations in ERBB2 mutated tumors	
Type of mutations	n
ERBB2 only (no other mutation detected)	1
ERBB2 + KRAS	1
ERBB2 + TP53 + SMAD4	1
ERBB2 + PIK3CA + KRAS	1
Total ERBB2 mutated cases	4

Multiple gene mutations in NOTCH1 mutated tumors	
Type of mutations	n
NOTCH1 only (no other mutation detected)	0
NOTCH1 + ERBB4 + PIK3CA + PTEN + KRAS + TP53	1
Total NOTCH1 mutated cases	1

Multiple gene mutations in ALK mutated tumors	
Type of mutations	n
ALK only (no other mutation detected)	0
ALK + TP53	1
Total ALK mutated cases	1

Multiple gene mutations in MAP2K1 mutated tumors	
Type of mutations	n
MAP2K1 only (no other mutation detected)	0
MAP2K1 + PIK3CA	1
Total MAP2K1 mutated cases	1