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

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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, ionsemiconductor 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 $K R A S$ and exons 2,3 and 4 of $N R A S$ ) also have negative predictive value. ${ }^{2}$ The extension of community KRAS testing to all $R A S$ mutations favoured the implementation of multitarget testing methodologies. Nextgeneration 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 $B R A F F^{4}$ 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 $100 \times$, minimum variant frequency of $5 \%$ and confirmation by the Integrative Genomics Viewer visual inspection. Sequence variants, deemed real and reportable

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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 $\geq 1$ 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 ( $\mathrm{n}=240 ; 38.8 \%$ ), KRAS ( $\mathrm{n}=247 ; 37.8 \%$ ), NRAS ( $\mathrm{n}=30 ; 4.6 \%$ ) and BRAF ( $\mathrm{n}=63$; 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 | $98 \ddagger(15 \%)$ |
| 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.
$\ddagger 1 / 98$ 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 ( $\mathrm{n}=3$ p.E746_E749delELRE; $\mathrm{n}=1$ 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 K 57 N 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 $(\mathrm{n}=4)$ or BRAF $(\mathrm{n}=3)$ 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 $(\mathrm{n}=7$ ) and associated with KRAS ( $\mathrm{n}=20$ ).

## 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 $B R A F$ (mostly no V600E) mutations coexisted with an RAS gene alteration. ${ }^{7}$ The frequent association of PIK3CA mutations with the $R A S / R A F$ 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 $R A S / R A F$ 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 $H E R 2 / E G F R$ 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/ $m T O R$ pathway are potentially actionable. As an example, $A K T 1$ mutations were associated with primary resistance to anti-EGFR therapy. ${ }^{14}$ In our study, $A K T 1$ 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 $\left(0.7 \%{ }^{15}\right.$ to $\left.6 \%{ }^{16}\right)$. 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 $R A F / M E K / E R K$ pathway by
either $\operatorname{KRAS}(\mathrm{n}=4)$ or BRAF $(\mathrm{n}=3)$ 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 5fluorouracil chemotherapy. ${ }^{223}$ 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 $m T O R$ inhibitor rapamycin, ${ }^{26}$ or to the histone deacetylase inhibitor MS-275. ${ }^{27}$ Noteworthy, as it was shown in previous reports $F B X W 7$ were often ( $51.2 \%$ ) associated with KRAS mutations. ${ }^{28} 29$ Interestingly, concurrent molecular aberrations can contribute to limited therapeutic efficacy of $m T O R$ inhibitors in the presence of $F B X W 7$ 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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## 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. I 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. I 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 $\mathrm{H} \& \mathrm{E}$ 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) $10 \mu \mathrm{~m}$-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 $30 \mu \mathrm{~L}$ 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 $500 \times$ 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 hg 19 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).

|  | Cr 1 | Cr 1 | Cr 2 | Cr 2 |
| :--- | :--- | :--- | :--- | :--- |
| patient | DDR2 | NRAS | ALK | ERBB4 |$\quad$ CTNNB1


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| 130 |  |
| 131 | G12V |
| 132 |  |
| 133 |  |
| 134 |  |
| 135 |  |
| 136 |  |
| 137 |  |
| 138 |  |
| 139 |  |

234
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264
265
266

S45F

267
268
269
270
271
272
273
274
275
276
277
278
279
280
405 G12D
518 G12D

564 Q61K
603 Q61K

| cr 3 | cr 4 | cr 4 | cr 7 | cr 7 |
| :--- | :--- | :--- | :--- | :--- |
| PIK3CA | FBXW7 | FGFR3 | BRAF | EGFR |

E542K

E542K

H1047R
G466E

D594G
R385C

E542K

H1047R
R385C

Q546P

R465H

R385C

R465H

E545K

G469E

V600E

S582L

H1047R

E545K
polyT ex20
delELRE

V600E

R465H

E542K

R465H

E545K

H1047L

Q546K

V600E

E545K
E542K
deIELRE
E545K

H1047R
R776H

R278*

R385C

V600E

E542K
E542K

E542K

E545K

E545K

E545K

E545K
R385C

N581S
V600E

N51S

Q546H
E542K

E545K

T599

V600E

V600E

E542K

Q546R

E545K

R385C
V600E

V600E
$\mathrm{E} 542 \mathrm{~K}+\mathrm{H} 1047 \mathrm{Y}$

H1047R
E545K

E542K

R465H
V600E

G469R

R385C
V600E

E545K
R266C

V600E

|  |  | V600E |
| :--- | :--- | :--- |
| Q266C |  |  |
|  | R266C | V600E |

E545K

E545K

H1047L

E545K

H1047R

H1047R

V600E V600E

E545K

E545K
R465H

R505L

H1047R

M1043I

H1047R
H1047R

T1025A

Q546K

N581S
R266C

H1047L

E545K

E542K

## R266C

G466V

R266C

R385C

R385C
V600E

H1047R

E545K

E545K
polyT ex20

T1025A

R266C

V600E

S582L
G469A

R278*

| E545K |  |
| :--- | :--- |
| G1049R |  |
|  |  |
| E542K | R4600E |



V600E
V600E

N581S

H1047R
N581S

G466V

E545K

Y1021C

V600E

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

```
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

```
G12C
```

G12V
G12R
G12D

G12A
G12V

|  | G12D |
| :---: | :---: |
|  | G12D |
|  | A146T |
|  | G12V |
|  | G12D |
|  | G12D |
|  | G13D |
|  | G12D |
|  | G12D |
|  | K117N |
|  | Q61L |
| L57fs* |  |
| D252Y | A146T |
|  | G12V |
|  | G12V |
|  | G12D |
|  | G12D |
|  | G13D |


| T321fs* |  |
| :--- | :--- |
|  | G12D |
|  | G12A |
|  | G12C |
| p.? | G13D |
|  |  |
|  | G12S |
|  | G12S |
|  | A146T |

## 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

|  | G12V <br> G13D <br> G12V |
| :---: | :---: |
| P339S |  |
|  |  |
|  |  |
|  |  |
|  | G12 12 V |
| G12V |  |
| G12V |  |
| G12D |  |

G13D E17K

G12D
G12V

G12A

R173H A146T
G12V

G12D
G12V
G12D
G12V
G12D

G12D
G12D
G12D
G12D
G13D
G12V
G12V

V1578delV
D252Y
G12V
Q61H

G13D

G12V
G12V

G12A

L318fs*
G12S
G12D

G13D
G12V
G12V

G12A
G12D

G12D
G13D
A146P
G12V
L19F
G13D
G13D

G12D
G12V

G13D

G12C G12V

G13D

G12V

G12V
G12V

G13D
G12V

Q61L
A146T

K117N
G12D
G13D
G12C

G12D
G12D

G13D

G13C

## G12D

## G13D

G12C

G12D

A146T
p.?

G12S
G12V

A146T

A146T

E242*
A146T
G13D + G12V

G13D + G12D

A59E

Q61H

G12V

L318fs*
K117N

Q61H

A59T

G12V

G12V

G12A

G12D
Q61H

G12D
A146T

A146V

G12S

G13D

S170N

```
                                    E17K
K117N
```

Q61L

R173H Q61L

A146T

K117N

Cr 17
cr 17
TP53
cr 18
SMAD4

R156fs*
E286fs* P281fs*
Y205H

P281fs*

Y234N

R175H

E198K

R306*

R342*
A276D
R175H

R283C

R175H

K132R

P278fs*

Q104*
R213fs*

R65H


#### Abstract

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
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*

H179Q

R65H

R342* + P295S

C238Y

N239S
R209fs*

C229fs*

R175H

K132R

R175H

R175H
I251fs*
I254S

R283H

C238Y

G168*

I195T
R175H
R361H

R196*

R175H

K132Q

A118V

R175H

R342*

E271K
R65H

R175H
H193Y

C242fs*
C242fs*
p.?

P278R

I255S
R213*

G245V
S183*

R65H
R175H

V80M

I195S
R213*
R306*

R175H

C176F

R175H
p.?

S94*

T231I

V842I

R175H

1232S

R175H

R280G
R175H

R110C
I195T
R267W

E204*
N288fs*
R156fs*

S215G

Y220C
R175H
p.?

Y236N
R175H
R175H
P281fs*

R306*
R361H
C135F

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.?

I195T

V8421 | R306* |  |
| :--- | :--- |
|  | p.? |
|  | R175H |
| R175H |  |

R65H

R213*

P190L

C176F

R306*

N131delN

V80M
R249M
A159D
R306*

V274F

P152L

| cr 1 | cr 1 | cr 2 | cr 2 | cr 3 |
| :--- | :--- | :--- | :--- | :--- |
| DDR2 | NRAS | ALK | ERBB4 | CTNNB1 |


| cr 3 | cr 4 | cr 4 | cr 7 | cr 7 |
| :--- | :--- | :--- | :--- | :--- |
| PIK3CA | FBXW7 | FGFR3 | BRAF | EGFR |

F384L

F384L

F384L

F384L

F384L

F384L

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

R173C

N375S

E168D

N375S

N375S

N375S

N375S

N375S

N375S

R173C

| cr 17 | cr 17 | cr 18 | cr 19 |
| :--- | :--- | :--- | :--- |
| ERBB2 | TP53 | SMAD4 | STK11 |

G245S

R273H + R213Q

R248W

R273C

R248Q
R282W

R273C

R282W

R273H

F354L

R248W
Y236D

R361C

R248W

V272M
R248Q
R273H

G245D

R273C

R273C

R273C

G245C

R273H

Y163C

R273H
R361C

R282W

## R248W

C176Y

R273C

G245S

R248Q
R361C

R267Q

R248Q
R248Q

F354L

R248W
R248W
R282W

R361C

G245S

R248Q

R248W
M237I

V272M

P281L

R248Q
R273H

| R273H | R361C |
| :--- | :--- |
| C275Y |  |
| R337C | R361C |
| H193L | R361C |

R337C
R361C

D208V

## R273H

G245D R361C

Y126D

V216M

R282W
R248W

S127F + S99F
R273C

G245S
G244D
R273H

R273C

R273H

R273H

R273H
R248W

R282W

R249K
G244D

R248Q

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

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

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 | 247 |
| KRAS + + ERBB4 + PIK3CA + NOTCH1 + PTEN + TP53 | 1 |
| Total KRAS mutated cases |  |
| 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 |

