Romina Oliveira Silva

Mutational analysis of *KRAS* and *NRAS* in metastatic colorectal cancer

Dissertação de candidatura ao grau de **Mestre em Oncologia** – especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador: Manuel António Rodrigues Teixeira, MD, PhD
Diretor do Serviço de Genética e Centro de Investigação
Instituto Português de Oncologia do Porto

Professor Catedrático Convidado do Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas de Abel Salazar – Universidade do Porto

Coorientador: Isabel Maria da Silva Veiga dos Santos, MSc
Assessora da carreira dos técnicos superiores de saúde, no ramo de Genética, no Serviço de Genética
Instituto Português de Oncologia do Porto

“The future belongs to those who believe in the beauty of their dreams.”

Eleanor Roosevelt

AGRADECIMENTOS

Ao meu orientador, Prof. Manuel Teixeira, por me ter dado a oportunidade de realizar este trabalho no seu grupo de investigação. Obrigada por toda a paciência, disponibilidade e orientação que contribuíram para o meu desenvolvimento enquanto investigadora.

À minha coorientadora, Isabel, por ter partilhado comigo toda a sua experiência e conhecimento e por me ter ajudado a superar todos os obstáculos que se foram colocando no meu caminho, sempre com boa disposição e alegria.

À Prof. Berta, atual diretora do Mestrado, e a todos os docentes por terem partilhado connosco todo o seu conhecimento sobre esta vasta área que é a Oncologia.

A todo o Serviço de Genética do IPO-Porto, por me terem recebido da melhor forma possível e por toda a força que me deram ao longo desta etapa. Um agradecimento especial à Anita e à Susana Bizarro por terem acreditado em mim e nas minhas capacidades; à Catarina, à Paula, ao Henrique, à Sara e ao Rui por me terem proporcionado as melhores horas de almoço de que tenho memória, sempre com as histórias mais hilariantes e as risadas mais contagiantes; e à Carla, à Manuela e à Patrícia por terem sido os meus "anjos da guarda" durante toda esta fase, por me terem encorajado quando me sentia prestes a desistir e por me terem ajudado quando eu não sabia que precisava de ajuda.

À Catarina Araújo, pela ajuda, compreensão e companheirismo ao longo deste percurso. Por se apresentar todos os dias com um sorriso na cara e carinho em cada abraço. Obrigada, principalmente, por me mostrares que as melhores amizades podem surgir quando menos esperamos.

Às meninas da molecular do GDPN: Marta, Diana, Cláudia Martins, Natália, Isa, Liliana, Elsa e Ariana. Obrigada por me terem integrado logo no vosso grupo, por toda a paciência com os meus horários malucos e por todo o apoio nesta fase mais complicada da minha vida.

A todos aqueles que contribuíram para que nos últimos 5 anos eu tivesse a melhor experiência académica que poderia ter tido. Um agradecimento especial aos amigos que fiz durante a licenciatura que, de uma forma ou de outra, estarão sempre comigo para onde quer que eu vá; aos “Sacaninhas da Presidente”, por terem sido os meus companheiros nesta aventura e por toda a diversão, risota e momentos partilhados ao longo destes dois anos; e às meninas da Residência, por me terem recebido de braços abertos e me terem proporcionado uma experiência que nunca esquecerei, sempre com um pouco de loucura e boa disposição à mistura.

Aos meus 11 magníficos: Ana, Carina, Carlos, Cati, Jeje, Joana, João, Márcia, Mari, TG e Ritinha. Obrigada por toda a cumplicidade, amizade, loucura, diversão, carinho, compreensão, dedicação, união, paciência, afeto e presença constante. Por aturarem o pior e aceitarem o melhor que há em mim. Já nada é, nem nunca mais será, o mesmo sem vocês.

Ao Chris e à Inês, dos melhores amigos que a vida me poderia ter dado, pela força, pelos sorrisos, pela brincadeira e pela confiança. Mas, principalmente, por me terem dado a oportunidade de provar que a desculpa de que o tempo e a distância destroem amizades, é apenas isso, uma desculpa.

À minha família, que sempre me acompanhou e esteve presente em cada etapa da minha vida, e esta não foi exceção. Obrigada por todo o apoio e força que, diariamente, me fazem alcançar todos os meus objetivos e superar todas as adversidades.

Às minhas irmãs, os meus caramelos repetidos, que tantas dores de cabeça me dão mas sem as quais a minha vida não seria a mesma. Obrigada por todas as “cusquices”, risotas e palermices, mas principalmente, por tornarem a minha vida tudo menos monótona.

Aos meus pais, a quem devo tudo aquilo que sou e aos quais não tenho palavras suficientes para agradecer tudo o que me foram proporcionando ao longo da vida. Obrigada por todas as palavras de encorajamento, pela força e apoio incondicionais ao longo de cada etapa e decisão da minha vida. Obrigada por simplesmente estarem lá para me ajudarem a tornar-me na melhor pessoa que posso ser. Este trabalho é dedicado a eles.

ABSTRACT

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that, upon activation, triggers several pathways, such as the RAS/RAF/ERK pathway, that are often deregulated in colorectal carcinomas. Two monoclonal antibodies targeting EGFR, cetuximab and panitumumab, have proven to be effective in the treatment of metastatic colorectal cancer (mCRC). However, it was discovered that patients with activating mutations in exon 2 (codons 12/13) of the *KRAS* gene do not respond to this therapy, establishing them as the first negative predictors of response to anti-EGFR therapy.

The *KRAS* gene, an effector of EGFR signaling through the RAS/RAF/ERK pathway, is mutated in exon 2 in about 40% of all mCRC. In *KRAS* exon 2 wild-type patients, only 40 to 60% achieve a response when receiving this therapy, something that suggests that alterations in other EGFR downstream effectors may also be associated with the lack of response to this therapy. Recently, the importance of less frequent *KRAS* and *NRAS* (RAS) mutations has been uncovered based on new results from recent clinical trials, which reported that patients with rarer activating RAS mutations also do not benefit from anti-EGFR therapy. This information shows the need to identify new predictive biomarkers that might help to select patients who are most likely to benefit from anti-EGFR.

In a consecutive series of 241 mCRC samples, wild-type for *KRAS* codons 12 and 13, we searched for less frequent RAS mutations that might act as predictor of response to anti-EGFR therapy, namely mutations in less frequent mutational hotspots in *KRAS* (exon 3 and 4) and *NRAS* (exons 2, 3 and 4).

About 19% (46/241) of the cases evaluated had a mutation in the analyzed regions. All mutations were found in heteregozity and were mutually exclusive. Thirty cases (12.4%; 30/241) had a *KRAS* mutation and sixteen (6.6%; 16/241) had a *NRAS* mutation, with the following distribution: thirteen mutations were found in *KRAS* exon 3 (28.3%; 13/46), seventeen in *KRAS* exon 4 (37.0%; 17/46), eight in *NRAS* exon 2 (17.4%; 8/46) and eight in *NRAS* exon 3 (17.4%; 8/46). No mutations were found in exon 4 of the *NRAS* gene. One novel point mutation, not previously described in CRC, was found in exon 3 of the *NRAS* gene in two cases. *KRAS* mutations were more frequent in earlier than in later stages at diagnosis (P=0.001).

In conclusion, nearly one-fifth of mCRC patients wild-type for *KRAS* exon 2 (codons 12/13) present other, less frequent, RAS mutations that might be associated with lack of response to anti-EGFR therapy. However, further studies are necessary to confirm these mutations as negative predictors of response to this therapy in the patients of our series.

RESUMO

O recetor de fator de crescimento epidérmico (EGFR) é uma tirosina cinase transmembranar que, após ativação, inicia diversas vias de transdução de sinal, tais como a via RAS/RAF/ERK, cuja atividade está frequentemente desregulada em carcinomas colo-retais. Dois anticorpos monoclonais dirigidos ao EGFR, cetuximab e panitumumab, provaram ser eficazes no tratamento do cancro colo-retal metastático. No entanto, foi descoberto que indivíduos com mutações ativantes nos codões 12 e 13 do gene *KRAS* não beneficiam deste tratamento, o que as torna no primeiro biomarcador preditivo de ausência de resposta à terapia anti-EGFR.

O gene *KRAS*, um efetor do EGFR através da via de transdução de sinal RAS/RAF/ERK, está mutado no exão 2 em cerca de 40% dos carcinomas colo-retais metastáticos. Dos doentes sem mutação no exão 2 do *KRAS*, só 40 a 60% respondem ao tratamento, o que sugere que alterações noutras proteínas efetoras do EGFR poderão estar envolvidas nesta ausência de resposta. Recentemente, foi revelada a importância de mutações menos frequentes nos genes *KRAS* e *NRAS* (RAS) em ensaios clínicos, que reportaram que indivíduos com estas mutações não beneficiam do tratamento com anti-EGFR.

Numa série consecutiva de 241 casos de carcinoma colo-retal metastático, sem mutações nos codões 12 e 13 do *KRAS*, procurámos mutações RAS menos frequentes que possam ser usadas como biomarcadores preditivos de resposta à terapia com cetuximab e panitumumab. Em particular, foram pesquisadas mutações noutros codões do *KRAS* (exões 3 e 4) e do *NRAS* (exões 2, 3 e 4).

Cerca de 19% (46/241) dos casos analisados apresentavam uma mutação nas regiões analisadas. Todas as mutações foram encontradas em heterozigotia e eram mutuamente exclusivas. Trinta casos (12,4%; 30/241) tinham uma mutação no *KRAS* e dezasseis (6,6%; 16/241) no *NRAS*, com a seguinte distribuição: treze mutações no exão 3 (28,3%; 13/46) e dezassete no exão 4 (37,0%; 17/46) do *KRAS*; oito no exão 2 (17,4%; 8/46) e oito no exão 3 (17,4%; 8/46) do *NRAS*. Não foram encontradas mutações no exão 4 do gene *NRAS*. Uma mutação pontual nova, não descrita em cancro colo-retal, foi encontrada no exão 3 do *NRAS* em dois casos. As mutações no *KRAS* foram mais frequentes nos estádios mais precoces do que nos mais tardios na altura do diagnóstico ($p=0,001$).

Em conclusão, aproximadamente um quinto dos doentes com cancro colo-retal metastático, sem mutações nos codões 12 e 13 do *KRAS*, apresentam outras mutações RAS menos frequentes que podem estar associadas à ausência de resposta à terapia

anti-EGFR. No entanto, são necessários estudos adicionais para confirmar o papel destas mutações como biomarcadores preditivos de ausência de resposta a esta terapia nos doentes da nossa série.

TABLE OF CONTENTS

FIGURE INDEX IX

TABLE INDEX XI

RELEVANT ABREVIATIONS XIII

I. INTRODUCTION 3

EPIDEMIOLOGY 3
RISK FACTORS 5
CRC DIAGNOSIS AND STAGING 6
CRC TREATMENT 8
COLORECTAL CARCINOGENESIS 10
CHROMOSOMAL INSTABILITY (CIN) PATHWAY 12
THE WNT SIGNALING PATHWAY 12
RAS PATHWAY 13
TP53 13
OTHER PATHWAYS INVOLVED IN CIN 14
MICROSATELLITE INSTABILITY (MSI) PATHWAY 14
CPG ISLAND METHYLATOR PHENOTYPE (CIMP) 15
KRAS AND *NRAS* MUTATIONAL STATUS AND ITS IMPORTANCE IN CRC TREATMENT 16
EGFR SIGNALING PATHWAYS IN CRC 16
THE RAS/RAF/ERK PATHWAY 17
PREDICTIVE BIOMARKERS OF ANTI-EGFR THERAPY RESPONSE 19

II. AIMS OF THE STUDY 25

III. MATERIALS AND METHODS 29

SAMPLES 29
DNA EXTRACTION FROM FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE 30
MUTATIONAL STATUS ANALYSIS 30
HIGH RESOLUTION MELTING 30
DNA SEQUENCING 32
STATISTICAL ANALYSIS 32

IV. RESULTS 35

MUTATIONAL TYPE AND DISTRIBUTION 35
NOVEL MUTATIONS 37
CLINICOPATHOLOGICAL ASSOCIATIONS 38

V. DISCUSSION 45

VI. CONCLUSIONS 53

VII. FUTURE PERSPECTIVES 57

VIII. REFERENCES 61

FIGURE INDEX

Figure 1. Estimated age-standardised incidence and mortality rates of CRC for male and female in the world. 4

Figure 2. Estimated age-standardised incidence and mortality rates of CRC for male and female in (A) Europe and (B) Portugal. 5

Figure 3. Circle graph depicting the genotypic and phenotypic heterogeneity in CRC. 6

Figure 4. Different steps of the adenoma-carcinoma sequence and the alterations associated with each one. 11

Figure 5. Wnt-pathway in the presence of A) wild-type and B) mutated *APC*. 13

Figure 6. Genetic instability pathways and their overlapping relationships. 16

Figure 7. EGFR signaling pathways 17

Figure 8. Upstream signaling of RAS and its control by the GDP-GTP cycle. 18

Figure 9. Oncogenic activation of the RAS/RAF/ERK pathway. 19

Figure 10. High resolution melting analysis of *KRAS* exon 3. A) Normalized and B) difference graphs, with wild-type (blue) and mutated (green and red) samples. 31

Figure 11. Electropherogram of *KRAS* exon 3 sequence, with A) wild-type and B) mutated sample. 32

Figure 12. Distribution (%) of the 46 mutations detected in all analyzed exons in mCRC samples. 36

Figure 13. Electropherograms of the mutation found in *NRAS* exon 3 that was not previously described, with A) wild-type and B) mutant sample. 37

Figure 14. Mutational status (%) in the subgroup of cases with available clinical data. 38

Figure 15. Distribution of *KRAS* and *NRAS* mutations according to patient sex. 39

Figure 16. Distribution of *KRAS* and *NRAS* mutations according to patient age at diagnosis. 40

Figure 17. Distribution of *KRAS* and *NRAS* mutations according to patient stage at diagnosis. 41

Figure 18. Distribution of *KRAS* and *NRAS* mutations according to primary tumor site. 42

TABLE INDEX

Table 1. AJCC cancer stanging for colon and rectal carcinomas. ___________________ 7

Table 2. Staging groups for CRC, according to AJCC guidelines.___________________ 8

Table 3. Design characteristics used in A) meta-analysis and B) the change in median progression-free survival in *KRAS* wild-type and mutant groups of each study. ____ 20/21

Table 4. Components of the PCR reaction mixture. ____________________________ 30

Table 5. Mutational status of the 241 mCRC samples analyzed. _________________ 35

Table 6. *KRAS* mutations identified after automated sequencing._________________ 36

Table 7. *NRAS* mutations identified after automated sequencing. ________________ 37

Table 8. Mutational status in the subgroup of cases with available clinical data.______ 38

Table 9. Distribution of *KRAS* and *NRAS* mutations according to patient sex. _______ 39

Table 10. Distribution of *KRAS* and *NRAS* mutations according to patient age at diagnosis. ____ 40

Table 11. Distribution of *KRAS* and *NRAS* mutations according to patient stage at diagnosis. ____ 41

Table 12. Distribution of *KRAS* and *NRAS* mutations according to primary tumor site._ 42

RELEVANT ABREVIATIONS

***AKT* –** v-akt murine thymoma viral oncogene homolog

***APC* –** Adenomatous polyposis coli

***ARAF* –** v-raf murine sarcoma 3611 viral oncogene homolog

***BAX* –** BCL2-associated X protein

***BRAF* –** v-raf murine leukemia viral oncogene homolog B1

***CCND1* –** Cyclin D1 encoding gene

***CDK8* –** Cyclin-dependent kinase 8

***c-RAF1* –** v-raf-1 murine leukemia viral oncogene homolog 1

CRC – Colorectal cancer

***CTNNB1* –** Catenin beta 1

***DCC* –** Deleted in colorectal cancer

DNA – Deoxyribonucleic acid

dNTP – Deoxyribonucleotide triphosphate

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

***ERBB1* –** see *HER1*

***ERBB2* –** see *HER2*

***ERBB3* –** see *HER3*

***ERBB4* –** see *HER4*

ERK – elk-related tyrosine kinase

ERK1 – elk-related tyrosine kinase 1

ERK2 – elk-related tyrosine kinase 2

FISH – Fluorescent *in situ* hybridization

G-domain – Guanine nucleotide-binding domain

GDP – Guanosine diphosphate

G-protein – Guanine nucleotide-binding protein

Grb2 – Growth factor receptor-bound protein 2

GTP – Guanosine triphosphate

***HER1* –** Epidermal growth factor receptor

***HER2* –** v-erb-b2 erythroblastic leukemia viral oncogene homolog 2

***HER3* –** v-erb-b2 erythroblastic leukemia viral oncogene homolog 3

***HER4* –** v-erb-b2 erythroblastic leukemia viral oncogene homolog 4

***HRAS* –** v-H-ras Harvey rat sarcoma viral oncogene homolog

IgG1 – Immunoglobulin G subclass 1

IgG2 – Immunoglobulin G subclass 2
IGFIIR – Insulin-like growth factor 2 receptor
JAK – Janus kinase
***KRAS* –** Kirsten rat sarcoma-2 viral (v-Ki-ras2) oncogene
MAPK – Mitogen-activated protein kinase
mCRC – Metastatic colorectal cancer
MEK – Mitogen-activated protein kinase kinase
MEK1 – Mitogen-activated protein kinase kinase 1
MEK2 – Mitogen-activated protein kinase kinase 2
***MGMT* –** O(6)-methylguanine-DNA methyltransferase
***MLH1* –** mutL homolog 1
***MSH2* –** mutS homolog 2
***MSH3* –** mutS homolog 3
***MSH6* –** mutS homolog 6
mTor – Mechanistic target of rapamycin
***NRAS* –** Neuroblastoma RAS viral (v-ras) oncogene homolog
PCR – Polymerase chain reaction
PI3K – Phosphoinositide-3-kinase
***PIK3CA* –** Phosphoinositide-3-kinase, catalytic, alpha polypeptide
***PTEN* –** Phosphatase and tensin homolog
PLC_{γ} – Phospholipase C gamma
SH2 – Src homolog 2
***SMAD2* –** SMAD family member 2
***SMAD4* –** SMAD family member 4
SOS – Son of sevenless
STAT – Sterol O-acyltransferase 1
TCF – T cell-factor
***TGF-α* –** Transforming growth factor α
***TGF-β* –** Transforming growth factor β
***TGFβRII* –** Transforming growth factor β receptor type II
***TP53* –** Tumor protein p53
VEGF – Vascular endothelial growth factor
VEGFR – Vascular endothelial growth factor receptor

INTRODUCTION

I. INTRODUCTION

With over 14 million new cancer cases and 8.2 million cancer deaths estimated to have occurred in 2012 (IARC, 2013), cancer is among the leading causes of death in the world. The burden of this malignancy seems to be increasing in economic developing countries, mostly due to population aging and growth, as well as a result of an increasing adoption of cancer-associated behaviors, such as smoking (Jemal *et al.*, 2011). Despite increasing awareness, colorectal cancer (CRC) remains as one of the most common cancers worldwide.

Epidemiology

CRC is a major cause of morbidity and mortality throughout the world, with over 1.3 million new cases diagnosed in 2012 (Figure 1). Europe and North America are among the regions with the highest incidence rates for this type of cancer. This rate is rapidly increasing in several areas that are considered as low risk areas, such as Eastern Asia. This might be the reflection of changes in dietary and lifestyle factors associated with "westernization", like smoking or obesity. In contrast to these high incidence trends, the occurrence of this pathology seems to be decreasing in several parts of the world, including the United States, probably due to population screening schemes that allow early detection of CRC and removal of precancerous lesions (Jemal *et al.*, 2011; Ferlay *et al.*, 2013; IARC, 2013).

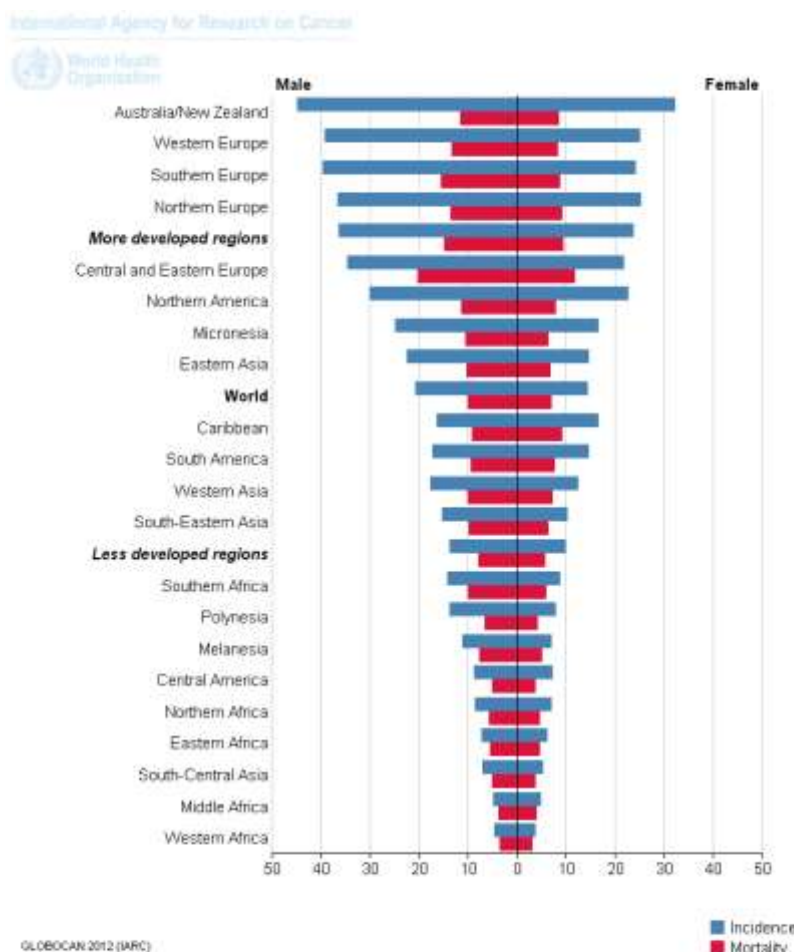


Figure 1. Estimated age-standardised incidence and mortality rates of CRC for male and female, in the world [Globocan, 2012 (IARC, 2013)].

In 2012, CRC was the second most common malignancy in Europe (excluding non-melanoma skin cancers), with 464.000 newly diagnosed cases estimated to have occurred, which accounts for 12.1% of all cancer cases. It had the third highest incidence in men, following prostate and lung cancer, and the second in women, only surpassed by breast cancer. This malignancy is slightly more incident in men than in women. It was also the second most frequent cause of death by cancer, with almost 215 000 deaths estimated, which accounts for 12.2% of all cancer deaths (Ferlay *et al.*, 2013).

In terms of CRC incidence and mortality, Portugal follows the same patterns of Europe (Figure 2). In 2012, it was the highest incident malignancy, with 7129 new cases diagnosed. Data analysis by sex demonstrated that CRC has the second highest incidence in both sexes, after prostate (male) and breast cancer (female). It was also the leading cause of death by cancer, with 3797 deaths, which accounts for 15.7% of all cancer deaths (Ferlay *et al.*, 2013; IARC, 2013).

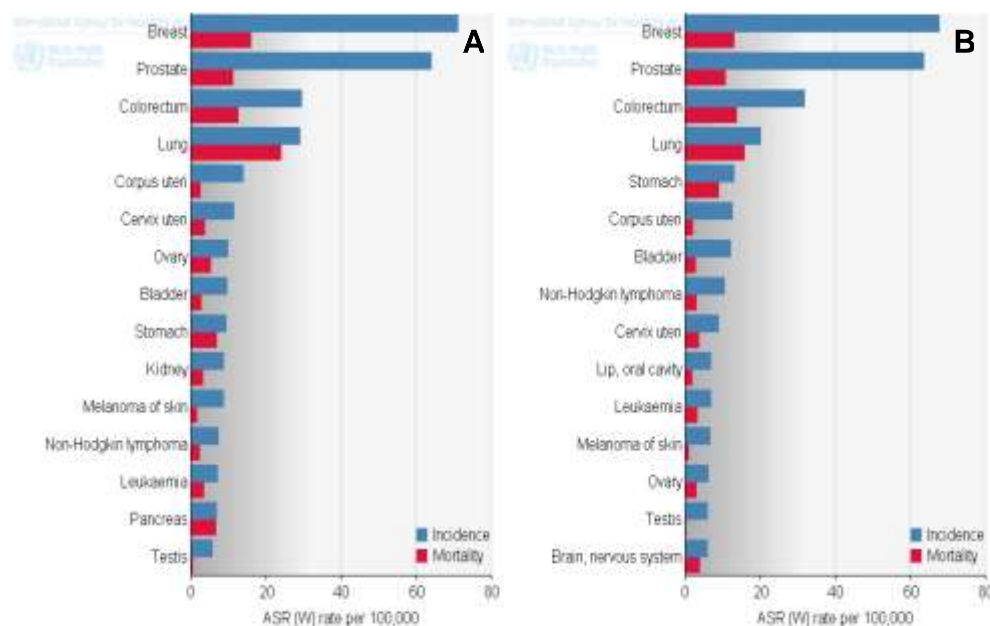


Figure 2. Estimated age-standardised incidence and mortality rates of CRC for male and female, in A) Europe and B) Portugal [Globocan, 2012 (IARC, 2013)].

Risk Factors

CRC is a very complex and heterogeneous disease and several etiologic factors contribute to the appearance of this malignancy.

The risk of developing CRC increases with age, preferentially after the age of 40. It is estimated that more than 90% of the patients diagnosed with this malignancy are aged 50 or older (Amersi *et al.*, 2005; Haggar & Boushey, 2009).

CRC usually occurs in one of three patterns: inherited, familial or sporadic. Inherited forms are responsible for about 5-10% of all CRC cancers, and are related to recognized hereditary conditions (Figure 3). The most common are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), also know as Lynch syndrome, which are responsible for 1% and 2%-4% of all CRC cases, respectively (Amersi *et al.*, 2005; Rustgi, 2007; Haggar & Boushey, 2009; Jasperson *et al.*, 2010). Other inherited diseases that lead to an increased risk of CRC are MUYTH-associated polyposis (MAP), Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS) (Rustgi, 2007; Jasperson *et al.*, 2010).

Familial cases are defined as families with increased predisposition to cancer, probably due to an hereditary basis with the involvement of genes that are less penetrant and/or the sign of shared environmental and lifestyle factors. It is estimated that about 20-30% of all CRC cases occur in this context (Rustgi, 2007; Jasperson *et al.*, 2010).

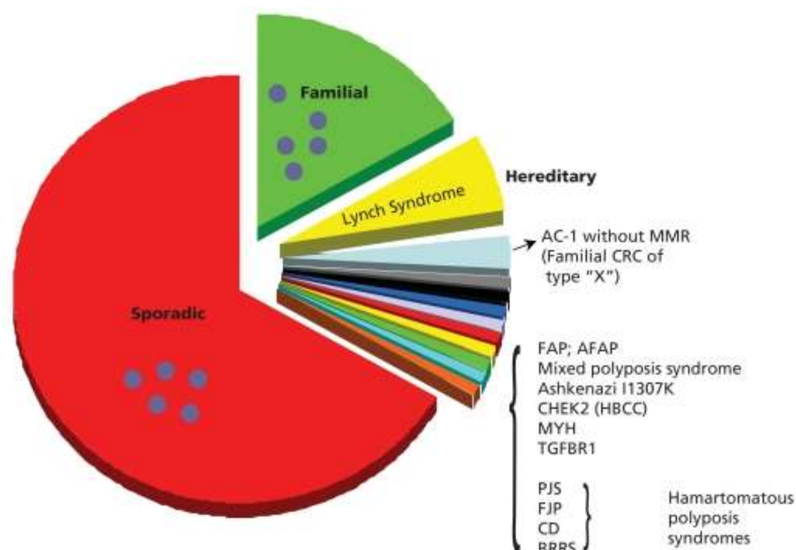


Figure 3. Circle graph depicting the genotypic and phenotypic heterogeneity in CRC [adapted from (Lynch *et al.*, 2009)].

Although genetics, family history and susceptibility factors play an important role in the development of this disease, the majority of CRCs are sporadic (~70%), with no prior family history (Haggar & Boushey, 2009). Several epidemiological studies have confirmed the influence of numerous environmental and dietary factors in the etiology of this disease, such as a diet high in fat and low in fiber, a sedentary lifestyle, obesity, diabetes, cigarette smoking and alcohol abuse (Haggar & Boushey, 2009; Chan & Giovannucci, 2010; Gingras & Beliveau, 2011; Colussi *et al.*, 2013). Physical activity, on the other hand, is thought to lower CRC risk. In fact, several studies report that higher overall levels of physical activity are associated with a decreased risk of CRC of ~20% (Huxley *et al.*, 2009). Another risk factor is the presence of chronic inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis (Ilyas *et al.*, 1999; Colussi *et al.*, 2013).

CRC Diagnosis and Staging

CRC diagnosis is usually made after the onset of the symptomatology of the disease, but most of the symptoms (rectal bleeding, blood in the stools, change in bowel habits) are non-specific and consistent with other conditions besides CRC. This, along with the fact that most patients with an early-stage disease are asymptomatic, presents difficulties when trying to diagnose this disease. The most common screening techniques are the fecal occult blood test and/or colonoscopy, the latter allowing direct inspection of the entire colon and same-session biopsy necessary for histopathological diagnosis. Patients should also undergo a physical examination and may perform computed tomography (CT), magnetic resonance imaging (MRI) or fluorodeoxyglucose-positron emission

tomography (FDG-PET), which allow the identification and characterization of a possible metastatic disease (Levin *et al.*, 2008; Van Cutsem, Nordlinger, *et al.*, 2010; Cummings & Cooper, 2011).

Once diagnosis is made, it is necessary to stage the tumor according to its pathological characteristics. Staging assessment is a key factor used to define treatment and to estimate the chance of a successful treatment outcome. The most common staging system for CRC is the TNM system (Centelles, 2012). Each of the three letters of the TNM system (Table 1), according to the American Joint Committee on Cancer (AJCC), stand for a specific meaning in regard to the characteristics of the tumor: T - size and/or extent (reach) of the primary tumor; N - amount of spread to nearby lymph nodes; and M - presence of metastasis or secondary tumors caused by the spread of cancer cells to other parts of the body (Edge *et al.*, 2010; Centelles, 2012). It is also important to report the timing of the staging assessment. Clinical staging – or cTNM – includes any information obtained before initiation of any kind of preoperative treatment. Pathological staging – pTNM – is defined by the information obtained after the examination of the surgically removed tissues (Edge *et al.*, 2010).

Table 1. AJCC cancer stanging for colon and rectal carcinomas [adapted from (Shia *et al.*, 2012)].

	Primary Tumor (T)
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades muscularis propria into pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral perinoteum
T4b	Tumor directly invades or is adherent to other organs or structures
	Regional Lymph Nodes (N)
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
	Distant Metastasis (M)
M0	No distant metastasis (no pathological M0; use clinical M to complete stage group)
M1	Distant metastasis
M1a	Metastasis confined to 1 organ or site (eg. liver, lung, ovary, nonregional node)
M1b	Metastases in more than 1 organ/site or the peritoneum

Based on TNM categories, cases with similar prognosis are grouped together in staging groups ranging from 0 to IV, with number IV representing the group with worse prognosis (Table 2).

Table 2. Staging groups for CRC, according to AJCC guidelines [adapted from (Centelles, 2012)].

	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1-T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage IIIA	T1-T2	N1	M0
	T1	N2a	M0
Stage IIIB	T3-T4	N1	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3-T4	N2b	M0
	T4b	N1-N2	M0
Stage IV	Any T	Any N	M1a
	Any T	Any N	M1b

CRC treatment

Among the different approaches used for the treatment of CRC, surgery is still the most common one, due to its curative intent. The goal of surgery is the complete resection of the tumor, which has a greater chance of success in localized diseases. After the resection, adjuvant therapy is recommended for stage III and "high-risk" stage II patients (Labianca *et al.*, 2010; Hagan *et al.*, 2013). However, about 20 to 25% of patients present metastatic disease at the time of the diagnosis, and 40 to 50% of newly diagnosed patients develop metastases over the course of the disease (Van Cutsem *et al.*, 2009). The majority of patients with metastatic colorectal cancer (mCRC) are not suitable for resection. However, in a small portion of cases, surgical resection can be achieved after the downsizing of the metastases with the administration of systemic therapy. On the other hand, patients with unresectable mCRC are subjected to systemic therapy with a palliative intent, rather than a curative one (Van Cutsem, Nordlinger, *et al.*, 2010; Edwards *et al.*, 2012).

Over the years, the standard chemotherapeutic (CT) regimens available evolved with the addition of several new therapeutic agents. For almost 40 years, 5-Fluorouracil (5-FU) was the only effective chemotherapeutic option for mCRC and nowadays it still remains a mainstay in mCRC treatment, in combination with other agents. This uracil analogue is converted into active metabolites, which inhibit the enzyme thymidylate synthase (TS).

This enzyme mediates the conversion of deoxyuridine monophosphate (dUMP) to deoxynucleotide triphosphates (dTMP), used in DNA synthesis. Its inhibition by 5-FU administration is then responsible for diminishing the availability of dTMPs, causing disruption of DNA synthesis and repair, which lead to cell death. 5-FU is usually administered with leucovorin (LV), a modulator that stabilizes the 5-FU-TS complex, increasing 5-FU cytotoxicity (Noordhuis *et al.*, 2004; Hirsch & Zafar, 2011). Due to the significant variation of its bioavailability when given orally, 5-FU can only be delivered in bolus or continuous infusion. This drawback led to the design of a prodrug (capecitabine) that can be administered orally and is equivalent to a continuous 5-FU infusion in terms of efficacy and overall survival of patients (Cassidy *et al.*, 2011; Hirsch & Zafar, 2011).

Besides 5-FU, two other agents – irinotecan and oxaliplatin – are currently used in standard combination CT regimens, which provide higher response rates and longer progression free survival, when compared with their administration as single agents (Van Cutsem, Nordlinger, *et al.*, 2010; Edwards *et al.*, 2012). Irinotecan is responsible for the inhibition of topoisomerase I, which causes irreversible single strand DNA breaks that lead to cell death. Oxaliplatin is a third generation platinum-based drug that acts by forming DNA adducts capable of restraining DNA replication and transcription. These agents may be administered in several combination regimens, such as FOLFOX (5-FU/LV/oxaliplatin), FOLFIRI (5-FU/LV/irinotecan) and CAPOX (capecitabine/oxaliplatin) (Lentz *et al.*, 2005; Van Cutsem, Nordlinger, *et al.*, 2010; Edwards *et al.*, 2012; NCCN, 2013).

Recent advances in the knowledge of the molecular pathology of tumors have led to the development of molecular targeted therapies, designed to interfere with specific molecules involved in carcinogenesis. In mCRC treatment, two molecules are targets of this kind of therapy: vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), with the former being targeted by bevacizumab and the latter by cetuximab and panitumumab. The introduction of these drugs in the treatment of mCRC patients resulted in a considerable improvement of median progression free survival (PFS) and response rates (RR) (Van Cutsem, Nordlinger, *et al.*, 2010; Edwards *et al.*, 2012).

Neoangiogenesis has been recognized for decades as a fundamental event in tumor growth and metastatic dissemination and the VEGF pathway is appointed as one of the major pathways involved in this process (Hicklin & Ellis, 2005; El Zouhairi *et al.*, 2011). VEGF is a proangiogenic factor overexpressed in several types of cancer, including CRC. It acts by binding to VEGF receptor (VEGFR) and triggering a cascade of different signaling pathways, such as proliferation and migration of endothelial cells and promotion of increased vascular permeability (Hicklin & Ellis, 2005; Kerbel, 2008). Bevacizumab is a recombinant humanized monoclonal antibody (moAb) that binds to VEGF and prevents it

from interacting with the receptors, thus inhibiting angiogenesis and altering vascular function and tumor blood flow. Bevacizumab as a single agent induces minimal response rates, however its true benefit lies in the combination with the traditional chemotherapy agents, since it normalizes the tumor vasculature, improving the delivery of anticancer agents to tumors (Ellis, 2006). It is now used as first-line treatment for mCRC, in combination with fluoropyrimidine-based CT regimens (Van Cutsem, Nordlinger, *et al.*, 2010; Edwards *et al.*, 2012; NCCN, 2013).

EGFR is a transmembrane receptor tyrosine kinase (TK) and a member of the ErbB family of receptor TKs whose abnormal activation is associated with uncontrolled cell proliferation, among other effects. EGFR is overexpressed on the surface of several epithelial tumors, including CRC (25 to 80% of cases) (Marshall, 2006). Cetuximab, a chimeric monoclonal IgG1, and panitumumab, a fully human IgG2, compete with EGFR's ligands and bind to the receptor, inhibiting activation of the downstream cell signaling pathways (Marshall, 2006; Ciardiello & Tortora, 2008). Both agents have been evaluated in several clinical trials, which resulted in their approval by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). At that time, it was also established as a guideline that all patients should be screened for *KRAS* mutations in codons 12 and 13 before anti-EGFR treatment, since these mutations were recognized as negative predictors of response to anti-EGFR therapy in CRC. Both moAbs are indicated for the treatment of mCRC *KRAS* wild-type in combination with fluoropyrimidine-, oxaliplatin- and irinotecan-containing CT regimens (Schmoll *et al.*, 2012; NCCN, 2013).

Colorectal carcinogenesis

The development of targeted therapies, which revolutionized mCRC treatment, was possible due to the growing understanding of the molecular mechanisms driving colorectal carcinogenesis. CRC evolves through a stepwise accumulation of several genetic and epigenetic alterations, which lead to the transformation of normal colonic mucosa into an invasive lesion. However, the identification of different molecular pathways of colorectal carcinogenesis has revealed the heterogeneity of this disease.

In 1990, Fearon and Vogelstein proposed the first genetic colorectal carcinogenesis model, the adenoma-carcinoma sequence model, which demonstrated the heterogeneous nature of CRC. This model was based upon four different features: **1)** Tumor arising depends on the activation of oncogenes coupled with the inactivation of tumor suppressor genes; **2)** Mutations in at least four to five genes are required for malignant transformation (fewer changes lead to benign lesions; **3)** When determining the tumor's biologic characteristics, total accumulation of changes is more important than the order in which

they occur; **4)** In some cases, mutant suppressor genes seem to exert a phenotypic effect, even in the heterozygous state, possibly meaning that not all tumor suppressor genes are "recessive" at the cellular level, like the *TP53* gene (Fearon & Vogelstein, 1990).

According to this model, the inactivation of the *APC* gene is described as the initiating event of colorectal carcinogenesis, leading to the formation of adenomas from normal colonical epithelium. Subsequent evolution of adenomas is frequently associated with activating mutations in *KRAS*, followed by allelic loss of chromosome 18q and inactivation of genes such as *DCC* or *SMAD2/4* (involved in the TGF-β pathway). Other genetic alterations, such as loss of heterozygosity (LOH) of chromosome 17p and inactivation of *TP53* gene, mediate the progression from adenoma to carcinoma (Figure 4) (Fearon & Vogelstein, 1990; Ilyas *et al.*, 1999; Worthley *et al.*, 2007; Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012). However, it is thought that only about 60% of the CRC cases follow the sequence highlighted by this model. This evidence suggests that other molecular modifications and/or pathways might be implicated in CRC carcinogenesis, and may lead to a more complete and refined model.

Currently, colorectal carcinogenesis is viewed as a result of the "genomic instability" phenomena, which denotes the loss of mechanisms involved in the maintenance of genomic fidelity and apoptosis that lead to the accumulation of alterations associated with colorectal tumorigenesis. Based on this theory, three distinct pathways were characterized: Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) (Worthley *et al.*, 2007; Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012).

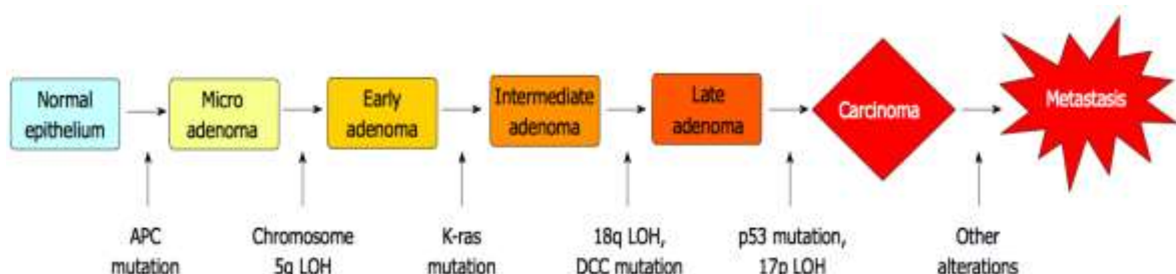


Figure 4. Different steps of the adenoma-carcinoma sequence and the alterations associated with each one [adapted from (Moran *et al.*, 2010)].

Chromosomal instability (CIN) pathway

The CIN pathway, also known as “suppressor” or “traditional” pathway, is the most common cause of genomic instability in CRC, encompassing 70-85% of all the sporadic CRC cases. It is characterized by the accumulation of numerical or structural chromosomal abnormalities that involve regions harboring genes crucial for the process of colorectal carcinogenesis (Worthley *et al.*, 2007; Markowitz & Bertagnolli, 2009; Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012). Frequent LOH at tumor suppressor gene loci and chromosomal rearrangements are characteristics of this type of tumors (Bogaert & Prenen, 2014). Alterations to the CIN pathway result from anomalies in chromosome segregation, with subsequent telomerase dysfunction/overexpression and defects in the DNA damage response mechanisms (Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012). The majority of the CIN tumors is located in the distal colon and is associated with poor prognosis (Kanthan *et al.*, 2012).

The Wnt signaling pathway

The “key” mutational event of this pathway occurs in the *APC* tumor suppressor gene. It is described as a “gatekeeper” gene of cellular proliferation in CRC that is associated with both sporadic CIN and, when mutated in the germline, the FAP syndrome (Ilyas *et al.*, 1999; Worthley *et al.*, 2007; Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013). This gene is involved in the Wnt signaling pathway and it is an important component of a degradation complex responsible for regulating β-catenin levels (Figure 5). APC inactivation impairs the normal degradation of β-catenin, leading to its cytoplasmic accumulation and eventual translocation into the nucleus, where it acts as a transcriptional co-activator of the TCF transcription factors family, affecting important cellular mechanisms, such as proliferation, differentiation and migration of normal cells (Worthley *et al.*, 2007; Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013). Cyclin D1 (*CCND1*) is one of the genes affected by this abnormal activation of the Wnt pathway. Increased *CCND1* regulation contributes to the development of this neoplasia by allowing the cell to evade apoptosis (Colussi *et al.*, 2013).

Other genetic alterations involved in β-catenin regulation include gain-of-function mutations in the β-catenin gene (*CTNNB1*), present in up to 50% of tumors lacking an *APC* mutation (Pino & Chung, 2010; Kanthan *et al.*, 2012; Colussi *et al.*, 2013) or *CDK8* gene amplification (Colussi *et al.*, 2013).

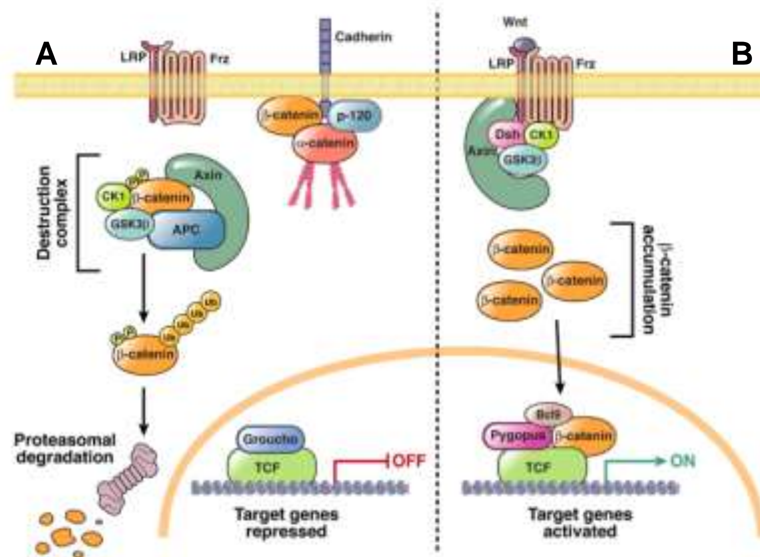


Figure 5. Wnt pathway in the presence of A) wild-type APC and B) mutated APC [adapted from (Pino & Chung, 2010)].

RAS pathway

KRAS gene mutations are one of the subsequent events to the early mutations above mentioned that lead to the progression from benign to malignant stages (Colussi *et al.*, 2013). *KRAS* is a proto-oncogene that encodes a GTP-binding protein, which is involved in the transduction and propagation of external signals. Somatic mutations in this gene, especially at exon 2, can cause a loss of inherent GTPase activity. This loss constitutively activates the mitogen-activated protein kinase (MAPK) signaling pathway, responsible for controlling cellular growth, survival, apoptosis, cell motility, differentiation and proliferation. This active state allows the cell to evade apoptosis and acquire a growth advantage (Ilyas *et al.*, 1999; Worthley *et al.*, 2007; Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013).

TP53

Alterations in the p53 tumor suppressor protein and its respective gene (*TP53*), localized in chromosome 17p, are common in most human cancers. In normal cells, the p53 protein, often designated as the “guardian” of the genome, is responsible for: 1) repairing DNA when a persistent damage occurs; 2) arresting cell cycle at the G1/S regulation point of DNA damage recognition; and 3) initiating apoptosis by inducing pro-apoptotic genes when DNA damage is irreparable (Worthley *et al.*, 2007; Kanthan *et al.*, 2012). Inactivation of *TP53* is, thereby, a key step in CRC development and is generally a late event in the traditional pathway. This inactivation is usually a combination of a missense mutation that inactivates the transcriptional activity of p53 and a 17p chromosome deletion of the second *TP53* allele (Markowitz & Bertagnolli, 2009; Kanthan *et al.*, 2012).

Other pathways involved in CIN

SMAD2, *SMAD4* and *DCC* genes are all located in the long arm of chromosome 18 (18q21.1). *SMAD2* and *SMAD4* are transcription factors involved in the TGF-β signaling pathway that regulates growth as well as apoptosis. The *DCC* gene codes for a large membrane receptor protein, from the immunoglobulin superfamily, that promotes apoptosis in the absence of its ligand (netrin-1) (Ilyas *et al.*, 1999; Worthley *et al.*, 2007). LOH of 18q is associated with negative prognosis and is reported in up to 60% of CRCs (Worthley *et al.*, 2007; Colussi *et al.*, 2013).

Mutations in the phosphoinositide-3 kinase gene (*PIK3CA*), detected in approximately a third of CRCs, often occur simultaneously with *APC* mutations, and cause increased AKT signaling even without the presence of growth factors. They also interact with a central regulator of cell growth and metabolism (mTOR) and with *KRAS*. Additionally, the phosphatase and tensin homolog (*PTEN*), which acts as a tumor suppressor gene in this pathway due to its inhibitory effect on PI3K-AKT signaling, is silenced in nearly 30% of CRC (Kanthan *et al.*, 2012; Yu *et al.*, 2014).

Microsatellite instability (MSI) pathway

Microsatellites are short repeat nucleotide sequences located throughout the genome, in both coding and non-coding regions. Because of their repetitive structure, they are prone to errors that occur during DNA replication. Those errors are recognized and repaired by the DNA Mismatch Repair (MMR) system during replication, which ensures a correct DNA synthesis (Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012). The MMR system is composed of multiple interacting proteins, such as MSH2 and MLH1, and mutations in the genes encoding these proteins lead to the inactivation of the MMR system and the accumulation of several DNA replication errors, resulting in MSI (Ilyas *et al.*, 1999; Markowitz & Bertagnolli, 2009; Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012).

MSI is the hallmark of the HNPCC syndrome, originated by MMR germline mutations. This pathway is also involved in the genesis of approximately 15% of sporadic CRC cases and is mostly caused by epigenetic silencing of the *MLH1* gene promoter (Al-Sohaily *et al.*, 2012; Kanthan *et al.*, 2012; Colussi *et al.*, 2013; Bogaert & Prenen, 2014). Tumors that develop through this particular pathway present a different phenotype from CIN positive CRCs: they are more likely to arise in the proximal colon, are poorly differentiated, often exhibit lymphocytic infiltration and, in general, patients affected by MSI-high (MSI-H) CRCs present better prognosis and survival. Sporadic MSI-H tumors are also characterized by a low frequency of *APC*, *CTNNB1* and *KRAS* mutations and a high

frequency of *BRAF* mutations, a member of the *RAF* family involved in the mediation of cellular response through the RAS-RAF-ERK pathway.

Several other genes, such as *TGFβRII* and *BAX*, are also mutated in these CRCs. *TGFβRII* inactivating mutations are found in more than 80% of all MSI-H CRCs and are involved in the adenoma transition to high-grade dysplasia or metastatic carcinoma. Mutations in the *SMAD2* and *SMAD4* genes, involved in the deactivation of TGFβ signaling, are also common in MSI-H CRCs. The pro-apoptotic tumor suppressor gene *BAX* is mutated in 50% of CRCs cases and allows tumor cells to evade the intrinsic apoptosis mechanisms. Additionally, mutations in other genes, such as *MSH3* and *MSH6*, Insulin Growth Factor Type 2 Receptor (*IGFIIR*) or *CCND1*, are also frequently present in MSI-H CRCs, although at a lower frequency then the ones mentioned above (Colussi *et al.*, 2013).

CpG island methylator phenotype (CIMP)

This third pathway, present in approximately 20 to 30% of CRC, consists of the aberrant hypermethylation of the CpG dinucleotide sequences localized in the promoter regions of genes involved in several functions, such as cell cycle regulation, apoptosis, DNA repair and invasion. This hypermethylation results in gene silencing, which provides an alternative mechanism for loss of function of tumor suppressor genes. In fact, the epigenetic silencing of a gene is biologically equivalent to acquiring an inactivating mutation, so it can occur as a first, second or both hits to inhibit gene expression (Worthley *et al.*, 2007; Markowitz & Bertagnolli, 2009; Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013).

CIMP tumors are classified as CIMP-high (CIMP-H) or CIMP-low (CIMP-L), based on the number of methylated markers (Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013). CIMP-H CRCs often contain *BRAF* gene mutations, which are associated with increased cell growth and progression of carcinogenesis. *BRAF* V600E (Val600Glu) mutation is present in 80 to 90% of CRC cases with sessile serrated adenomas (SSA) but is mostly absent in conventional adenomas. Additionally, *BRAF* mutations are present in early hyperplastic polyps (the serrated precursors) or in late dysplastic serrated adenomas that frequently have CIMP-H and MSI-H features, which leads to the hypothesis that the serrated pathway is involved in the sporadic CIMP CRCs development (Worthley *et al.*, 2007; Leggett & Whitehall, 2010; Colussi *et al.*, 2013).

Clinically, CIMP-H tumors have a particularly poor prognosis and are usually located in the proximal site of the colon, similar to MSI tumors (Worthley *et al.*, 2007; Bogaert & Prenen, 2014). On the other hand, CIMP-L tumors have a low level of DNA methylation

and, instead of the *BRAF* mutations observed in CIMP-H tumors, they are usually associated with *KRAS* and *MGMT* mutations (Worthley *et al.*, 2007; Colussi *et al.*, 2013; Bogaert & Prenen, 2014).

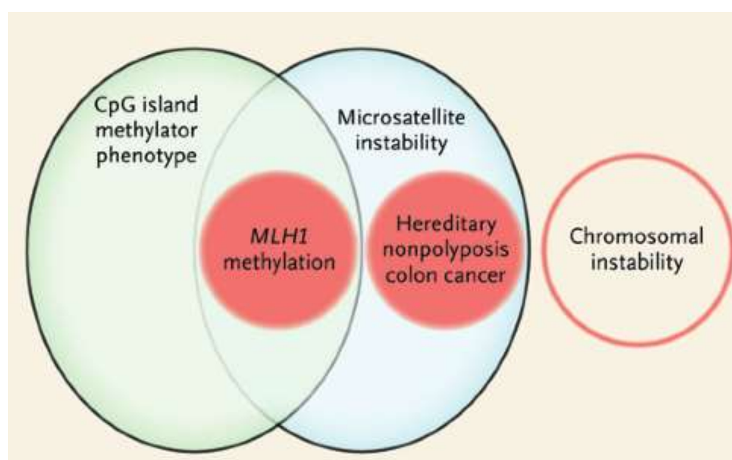


Figure 6. Genetic instability pathways and their overlapping relationships [adapted from (Markowitz & Bertagnolli, 2009)].

KRAS and *NRAS* mutational status and its importance in CRC treatment

The disclosure that patients with activating *KRAS* gene mutations do no benefit from anti-EGFR therapy (cetuximab or panitumumab) surfaced after a series of initial retrospective analyses. This made *KRAS* mutations emerge as the only negative biomarker predictor of response to this therapy. To better understand how *KRAS* activating mutations influence anti-EGFR therapy's efficacy, it is necessary to understand the link between EGFR and RAS in CRC.

EGFR signaling pathways in CRC

EGFR, also known as HER1/ERBB1, belongs to the ErbB family of receptor TKs, which comprises three other members: HER2 (ERBB2/neu), HER3 (ERBB3) and HER4 (ERBB4). All proteins of this family are anchored in the cytoplasmic membrane and share a similar structure composed by an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic TK-containing domain (Spano *et al.*, 2005; Normanno *et al.*, 2006; Scaltriti & Baselga, 2006). In normal cells, EGFR signaling pathway is activated in a ligand-dependent manner. ErbB family members can be activated by several known ligands, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), amphiregulin and epiregulin, that bind specifically to EGFR (Hynes & MacDonald, 2009).

EGFR activation by ligand binding induces the dimerization of the receptor with formation of homo- and heterodimers that leads to autophosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptors, initiating intracellular signaling via several pathways, namely RAS/RAF/ERK, PI3K/AKT, JAK/STAT and PLC$_\gamma$ (Figure 7) (Normanno *et al.*, 2006; Scaltriti & Baselga, 2006). These signal transduction cascades are responsible for diverse cellular responses, such as proliferation, migration, differentiation and apoptosis. Constitutional activation of these pathways can be achieved by receptor overexpression or activating mutations, which are common in several malignancies, including CRC (Normanno *et al.*, 2006; Roberts & Der, 2007).

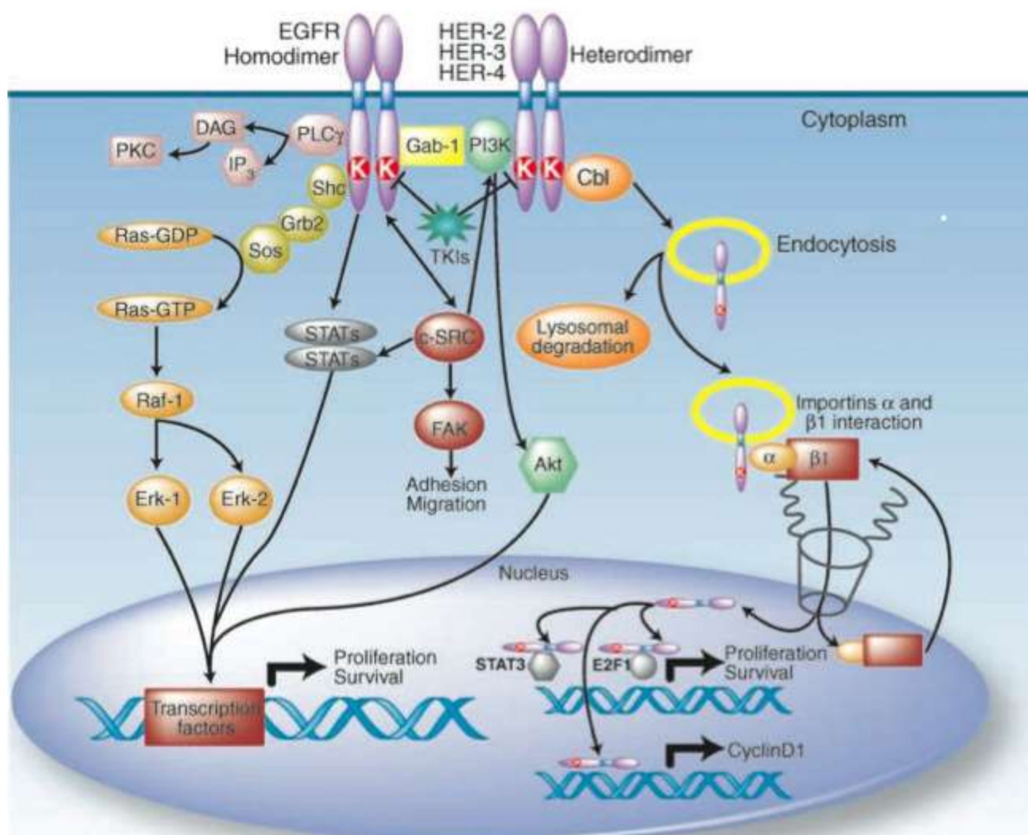


Figure 7. EGFR signaling pathways [adapted from (Scaltriti & Baselga, 2006)].

The RAS/RAF/ERK pathway

The RAS/RAF/ERK pathway is one of the most deregulated signaling pathways in human cancer. In this pathway, RAS activation leads to a sequential activation of three MAPKs (RAF, MEK and ERK), which in turn generate signals that promote regulation of several cellular responses that establish cell proliferation, survival and differentiation (Dhillon *et al.*, 2007; Roberts & Der, 2007).

RAS proteins – *KRAS*, *HRAS* and *NRAS* – are important components of a large family of small GTP-binding proteins. These three members of the RAS family are composed of a C-terminal and N-terminal regions, both important for RAS proteins functions. The C-terminal region contains a CAAX motif, which is the target of post-translational modifications that allow the recruitment of the proteins to the inner face of the plasma membrane, essential for their normal function (Downward, 2003; Karnoub & Weinberg, 2008). On the other hand, the N-terminal region is an important regulator of the protein GDP-bound and GTP-bound states. The structural differences between these states reside in two regions of the N-terminal, the switch I and switch II regions. Binding of the GTP molecule alters the conformation of both switch regions and allows the RAS protein to remain in an active state. Upon the release of the GTP's extra phosphate group, the switch regions modify their conformation and return to the inactive state (Karnoub & Weinberg, 2008; Santarpia *et al.*, 2012). This GDP/GTP cyclic process is catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), with the former facilitating the exchange from GDP to GTP and the latter promoting the hydrolysis of GTP to GDP (Figure 8). The balance between these proteins is important to determine the activation of the RAS protein and its downstream target pathways (Downward, 2003; Santarpia *et al.*, 2012).

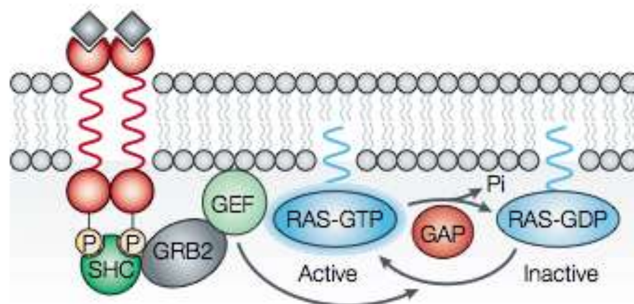


Figure 8. Upstream signaling of RAS and its control by the GDP-GTP cycle [adapted from (Downward, 2003)].

In normal cells, RAS becomes activated upon extracellular stimuli, which activate receptor TKs such as EGFR. The autophosphorylated receptor then binds to the SH2 domain of the adaptor protein growth-factor-receptor-bound protein 2 (GRB2). Since the SOS (GEF) is connected to the same adaptor protein in the SH3 domain, the binding of the receptor to the GRB2 brings SOS in close proximity to the RAS protein, leading to its activation (Downward, 2003; Roberts & Der, 2007). Activated RAS interacts with three closely related RAF kinases (c-RAF1, BRAF and ARAF), mobilizing them to the plasma membrane, where they become activated. Then, Raf kinases phosphorylate MEK1 and MEK2 (MAPKKs), which in turns triggers the phosphorylation and activation of MAPKs (ERK1 and ERK2) (Figure 9). Once activated, ERK1 and ERK2 are translocated to the

nucleus, where they regulate the activity of various transcription factors (Downward, 2003; Roberts & Der, 2007; Santarpia *et al.*, 2012).

Since this is a complex signaling pathway that ensures essential cellular responses, its deregulation is an important key factor to cancer progression. Several mechanisms, such as *KRAS* and *BRAF* activating mutations or EGFR overexpression, contribute to an improper activation of the pathway and have been described in several tumor types, including CRC. Furthermore, it was also identified that Erk activation can induce upregulation of EGFR ligands, which promotes an autocrine growth loop crucial for tumor growth (Roberts & Der, 2007; Santarpia *et al.*, 2012).

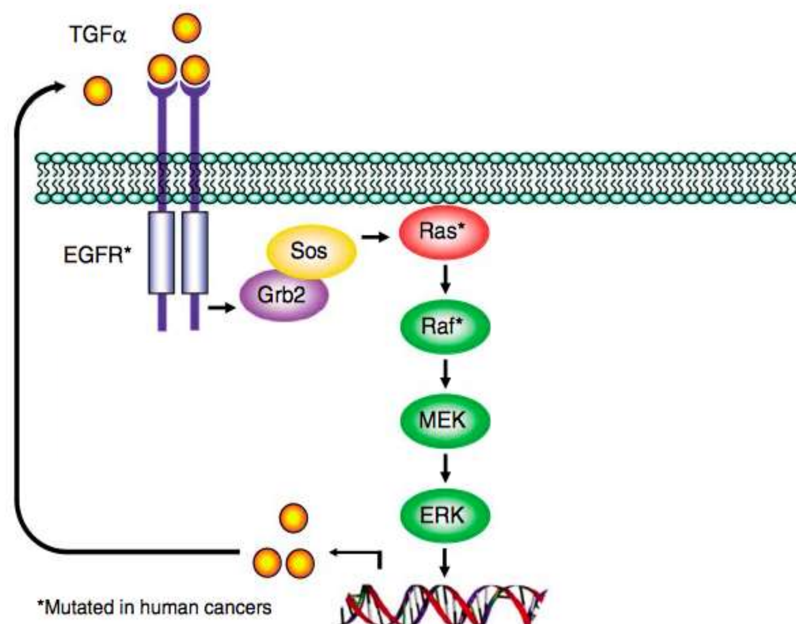


Figure 9. Oncogenic activation of the RAS/RAF/ERK signaling pathway [adapted from (Roberts & Der, 2007)].

Predictive biomarkers of anti-EGFR therapy response

As stated before, several retrospective studies of *KRAS* mutational status in tumors from patients treated with anti-EGFR therapy found that activating mutations in *KRAS* codons 12 and 13 were associated with a lack of response to these therapies (Table 3a and 3b). Mutations in these particular codons in *KRAS* exon 2, present in nearly 40% of all mCRC patients, cause constitutive activation of the RAS/ /ERK pathway, despite EGFR inhibition. Thus, screening for these mutations is recommended before therapy, since only patients with *KRAS* wild-type mCRC will benefit from it. However, among those patients with *KRAS* exon 2 wild-type tumors, only 40-60% responds to anti-EGFR therapy (De Roock *et al.*, 2008; Lievre *et al.*, 2008). This suggests that other activating mutations along this pathway may also confer resistance to anti-EGFR therapies. So, there is a

great need to identify alternative predictive biomarkers that will distinguish patients who are most likely to benefit from this type of therapy.

The first attempts to identify such biomarkers led to the discovery that positive EGFR overexpression (determined by immunohistochemistry) has no correlation with treatment response (Chung *et al.*, 2005) and that the association between increased EGFR gene copy number (detected by FISH) and treatment response remains uncertain/controversial (Moroni *et al.*, 2005; Laurent-Puig *et al.*, 2009). These findings make it difficult to establish EGFR alterations as predictive biomarkers for treatment response. However, several studies have been focusing in the analysis of other targets, such as other EGFR downstream effectors (*BRAF*, *NRAS*, and *PIK3CA*), as well as less frequent *KRAS* mutations (De Roock *et al.*, 2010; Guedes *et al.*, 2013).

Recent analyses of tumors from patients enrolled in clinical trials demonstrated that other *KRAS* (codons 59/61 – exon 3; codons 117/146 – exon 4) or *NRAS* (codons 12/13 – exon 2; codons 59/61 – exon 3; codons 117/146 – exon 4) mutations lead to increased levels of RAS-GTP. These recent studies also reported that most patients harboring these rarer mutations did not achieve an objective response with anti-EGFR therapy (Douillard *et al.*, 2013; Seymour *et al.*, 2013). Given this, recent guidelines for anti-EGFR CRC treatment recommend the evaluation of the mutational status of the three exons of both genes before treatment initiation with anti-EGFR therapy.

Table 3. Design characteristics used in A) meta-analysis and B) the change in median progression-free survival in *KRAS* wild-type and mutant groups of each study [adapted from (Adelstein *et al.*, 2011)].

Paper	Trial phase	Treatment comparisons	Line of therapy	Number of study participants				KRAS analysis	
				Overall	KRAS subgroup (% of total)	KRAS wild (% of KRAS)	KRAS mutant (% of KRAS)	Blinded	Codon
Monotherapy									
Amado[1]	3	BSC versus BSC + Pmab	3rd	463	427 (92.2)	243 (56.9)	184 (43.1)	Yes	12,13
Karapetis[2]	3	BSC versus BSC + Cmab	3rd	572	394 (68.9)	230 (58.3)	164 (41.6)	Yes	12,13
Irinotecan									
Van Cutsem[3]	3	FOLFIRI versus FOLFIRI + Cmab	1st	1198	540 (45.1)	348 (64.4)	192 (35.6)	ns	12,13
Van Cutsem[4]	Update				1063 (88.7)	666 (62.7)	397 (37.3)		
Peeters[5]	3	FOLFIRI versus FOLFIRI + Pmab	2nd	1186	1083 (91.3)	597 (55.1)	486 (44.9)	Yes	ns
Oxaliplatin									
Bokemeyer[6]	2	FOLFOX-4 versus FOLFOX-4 + Cmab	1st	337	233 (69.1)	134 (57.5)	99 (42.5)	ns	12,13
Bokemeyer[7]	Update				315 (93.4)	179 (56.8)	136 (43.2)		
Maughan[8]	3	Ox, 5FU versus Ox, 5FU + Cmab[b]	1st	1630	1316 (80.7)	729 (55.4)	565 (42.9)	ns	12,13,61
Douillard[9]	3	FOLFOX4 versus FOLFOX4 + Pmab	1st	1183	1096 (92.6)	656 (59.9)	440 (40.1)	Yes	ns
Tveit[10]	3	FLOX versus FLOX + Cmab[b]	1st	566	498 (87.9)	303 (60.8)	195 (39.2)	ns	ns
Oxaliplatin/Irinotecan + Bevacizumab									
Hecht[a,11] (Ox)	3B	Ox-CT/Bev versus Ox-Ct/Bev + Pmab	1st	823	664 (80.7)	404 (60.8)	260 (39.1)	ns	ns
Hecht[a,11] (Iri)	3B	Iri-CT/Bev versus Iri-CT/Bev + Pmab	1st	230	201 (87.3)	115 (57.2)	86 (43.0)	ns	ns
Tol[12]	3	Cap, Ox, Bev versus Cap, Ox, Bev + Cmab	1st	736	520 (70.6)	314 (60.3)	206 (39.6)	ns	12,13

BSC, Best supportive care; Cmab, cetuximab; Pmab, panitumumab; Iri, irinotecan; Ox, oxaliplatin; Ox-CT = oxaliplatin based chemotherapy; Iri-CT, irinotecan based chemotherapy; Bev, bevacizumab; Cap, capecitebine; FOLFOX, fluorouracil + leucovorin + oxaliplatin; FOLFIRI, fluorouracil + leucovorin + irinotecan; FU, 5fluorouracil. ns, not stated in paper.

[a] Differing drug regimens described in one paper.

[b] Additional randomisation undertaken but not reported in this analysis.

A

	Median PFS (months) in KRAS wild group			Median PFS (months) in KRAS mutant group		
	With anti-EGFR antibody therapy	No anti-EGFR antibody therapy	Difference	With anti-EGFR antibody therapy	No anti-EGFR antibody therapy	Difference
Monotherapy						
Armado[1]	2.8	1.7	1.1	1.7	1.7	0.0
Karapetis[2]	3.7	1.9	1.8	1.8	1.8	0.0
Irinotecan						
Van Cutsem[4 a]	9.9	8.4	1.5	7.4	7.7	−0.3
Peeters[5]	5.9	3.9	2.0	5.0	4.9	0.1
Oxaliplatin						
Bokemeyer[7 a]	8.3	7.2	1.1	5.5	8.6	−3.1
Maughan[8]	8.6	8.6	0.0	NR	NR	NR
Douillard[9]	9.6	8.0	1.6	7.3	8.8	−1.5
Tveit[10]	7.9	8.7	−0.8	9.2	7.8	1.4
Bevacizumab						
Hecht: Iri[11]	10.0	12.5	−2.5	8.3	11.9	−3.6
Hecht: Ox[11]	9.8	11.5	−1.7	10.4	11.0	−0.6
Tol[12]	10.5	10.6	−0.1	8.1	12.5	−4.4

[a] Updated (abstract) results used, NR = not reported in paper.

B

[1](Amado *et al.*, 2008) [2](Karapetis *et al.*, 2008) [3](Van Cutsem *et al.*, 2009) [4](Van Cutsem, Lang, *et al.*, 2010) [5](Peeters *et al.*, 2010) [6](Bokemeyer *et al.*, 2009) [7](Bokemeyer *et al.*, 2011) [8](Maughan *et al.*, 2010) [9](Douillard *et al.*, 2010) [10](Tveit *et al.*, 2010) [11](Hecht *et al.*, 2009) [12](Tol *et al.*, 2009)

AIMS OF THE STUDY

II. AIMS OF THE STUDY

The aim of this study was to establish the type and frequency of other *KRAS* and *NRAS* mutations in a large consecutive series of mCRC wild-type for *KRAS* exon 2 (codons 12/13) in order to contribute for the characterization of Portuguese patients.

The specific aims were:

I) To analyze the mutational status in mCRC by high resolution melting and automated Sanger sequencing of the following genes and exons:

- **a.** *KRAS* exons 3 (codons 59/61) and 4 (codons 117/146);
- **b.** *NRAS* exons 2 (codons 12/13), 3 (codons 59/61) and 4 (codons 117/146);

II) To determine the frequency and type of mutations in each of the above genes and exons in Portuguese mCRC patients;

III) To establish associations between the tumor genetic alterations and clinicopathological features in mCRC patients;

MATERIALS AND METHODS

III. MATERIALS AND METHODS

Samples

A consecutive series of formalin-fixed paraffin embedded tumor samples from 400 patients eligible for anti-EGFR therapy were selected to be retrospectively analyzed. All these samples belong to patients referred to the Genetics Department of IPO-Porto between August 2008 and December 2012 for *KRAS* exon 2 (codons 12/13) mutation analysis. Of the 400 samples analyzed, 243 were considered wild-type by PCR or high resolution melting (HRM), followed SNaPshot and/or automated sequencing. In our study, those 243 *KRAS* exon 2 wild-type samples were analyzed for mutations in *KRAS* (codons 59/61 – exon 3; codons 117/146 – exon 4) or *NRAS* (codons 12/13 – exon 2; codons 59/61 – exon 3; codons 117/146 – exon 4). Of these 243 samples, 2 were excluded due to lack/poor quality DNA. 80 samples had previously been analyzed, for *KRAS* exon 3 (codons 59/61) and *KRAS* exon 4 (codon 146), by our group in another study (Guedes *et al.*, 2013). Of the final 241 cases analyzed, histopathology reports were available for 215 cases (66 women and 149 men). Median age of diagnosis was 58 years old and tumor localization was as follows: 23 ascending colon, 14 descending colon, 1 transverse colon, 62 sigmoid colon and 115 rectum tumors. This study was approved by the institutional review board of the Portuguese Oncology Institute - Porto.

DNA extraction from formalin-fixed paraffin-embedded tissue

Whenever possible, tumor areas containing at least 50% of tumor cells were delimited, by a pathologist, in the hematoxylin and eosin (H&E) stained slides of each sample. The corresponding unstained slides were immersed in xylene $_{[SIGMA]}$ and twice in ethanol 100% $_{[Merck]}$ for 5 minutes each. Tumor areas, which were previously delimited by comparison with the correspondent H&E stained slides, were macrodissected and transferred to a microcentrifuge tube. DNA was isolated using the QIAamp® DNA FFPE Tissue Kit $_{[QIAGEN]}$, following manufacturer's instructions. Finally, DNA was quantified by spectrophotometry with NanoDrop ND-1000® $_{[NanoDrop\ Technologies]}$.

Mutational status analysis

All samples were initially screened by HRM for mutations in *KRAS* (NM_004985) exons 3 and 4 and *NRAS* (NM_002524.4) exons 2, 3 and 4, followed by automated DNA Sanger sequencing of one strand (forward or reverse), in order to evaluate the presence/absence of DNA alterations. A second HRM was performed in all positive samples of the initial analysis, followed by automated DNA Sanger sequencing of both strands.

High Resolution Melting

PCR amplification and HRM analysis were both performed on a LightCycler-480 II Real-Time System $_{[Roche\ Diagnostics]}$. The PCR reaction mixture added to each well, of a 96 well plate, was composed of a pair of primers (forward and reverse), DNA of each sample and PCR reagents (Table 4). To prevent contamination and/or evaporation, 15µL of mineral oil were added to each well. The plate was then sealed with sealing film and centrifuged at 2000rpm for 2 minutes.

Table 4. Components of the PCR reaction mixture.

PCR reaction mixture components	
2,5x LightScanner® Master Mix $_{[Idaho]}$	4.0µL
Forward primer $_{[frilabo]}$	350nM
Reverse primer $_{[frilabo]}$	350nM
DNA	20-100ng
Reagent grade water $_{[Idaho]}$	4.9µL
Total reaction volume	10µL

The primer pairs used in this study for *NRAS* exons 2, 3 and 4 and *KRAS* 3 and 4 were all designed with primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and are available upon request to the Department of Genetics of IPO-Porto.

PCR amplification and HRM conditions were the same for all *NRAS* exons and for *KRAS* exon 3. *KRAS* exon 4 conditions differed from those applied to the exons mentioned above. However, all exons were subjected to an initial denaturation, followed by 35-40 cycles of amplification. After that, one cycle of heteroduplex and one of melting were done before the plate was cooled to 40ºC. The detailed conditions are described, separately, below:

I. For all *NRAS* exons and for *KRAS* exon 3: An initial denaturation at 95ºC for 15 minutes was followed by 35 cycles of 10 seconds at 95ºC, 20 seconds at 69ºC and 30 seconds at 72ºC. After that, one heteroduplex cycle was done at 97ºC for 1 minute and 40ºC for 2 minutes, followed by one melting cycle from 70ºC to 95ºC with 25acquisitions/ºC. The plate was finally cooled to 40ºC for 1 minute with a ramp rate of 2.2ºC/second.

II. For *KRAS* exon 4: Initial denaturation was done at 95ºC for 10 minutes and followed by 35 cycles of 20 seconds at 95ºC, 20 seconds at 65ºC and 20 seconds at 72ºC, with a final extension of 10 minutes at 72ºC. One heteroduplex and one melting cycle were done after that, with the samples being denatured with an initial hold of 5 minutes at 95ºC and 1 minute at 40ºC (heteroduplex cycle), followed by a melting profile from 70ºC to 90ºC with 25acquisitions/ºC (melting cycle). The plate was cooled to 40ºC in the same conditions as described before for all *NRAS* exons and *KRAS* exon 3.

Amplification and melting curves were obtained and analyzed using the LightCycler® 480 Gene Scanning software v1.5 [Roche diagnostics].

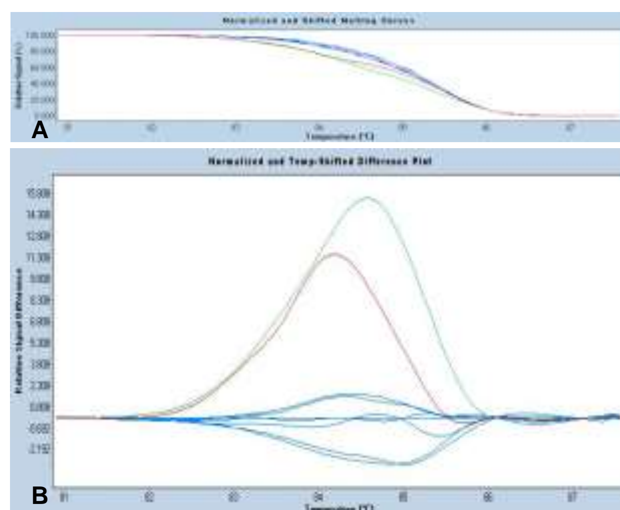


Figure 10. High resolution melting analysis of *KRAS* exon 3. A) Normalized and B) difference graph, with wild-type (blue) and mutated (green and red) samples.

DNA Sequencing

Before sequencing, all PCR amplification products were purified to remove excess of primers, salts, enzymes and dNTPs from the previous reaction. For that purpose, Illustra GFX PCR DNA and Gel Bad Purification Kit [GE Healthcare Life Sciences] and NZYGelpure Kit [nzytech] were used, according to the manufacturer's protocol.

After that, 1µL of each sample product was used for the sequencing reaction, which also contained 0.5µL of Big Dye® Terminator v1.1 cycle sequencing Ready Reaction Mix [Applied Biosystems], 3.4µL of Big Dye® Terminator v1.1, v1.3 5x sequencing buffer [Applied Biosystems], 350nM of one of the primers (forward or reverse) and 4.78µL of bidestilled sterile water [B. Braun], to a total volume of 10µL. Samples were then subjected to an initial denaturation at 95ºC for 4 minutes, followed by 35 cycles of 95ºC for 10 seconds, 50ºC for 10 seconds and 60ºC for 2 minutes, with a final extension of 60ºC for 10 minutes.

PCR sequencing products were purified using Illustra Sephadex® G-50 fine [GE Healthcare Life Sciences] and added to 12µL of Hi-Di™ Formamide [Applied Biosystems]. The products were then run in either an ABI PRISM™ 310 Genetic Analyzer [Applied Biosystems] or a 3500 Genetic Analyzer [Applied Biosystems]. Electropherograms of each sample were analyzed with the Sequencing Analysis Software v5.4 [Applied Biosystems]. All of them were read at least twice, reviewed manually and with the Mutation Surveyor Software v4.0.8.

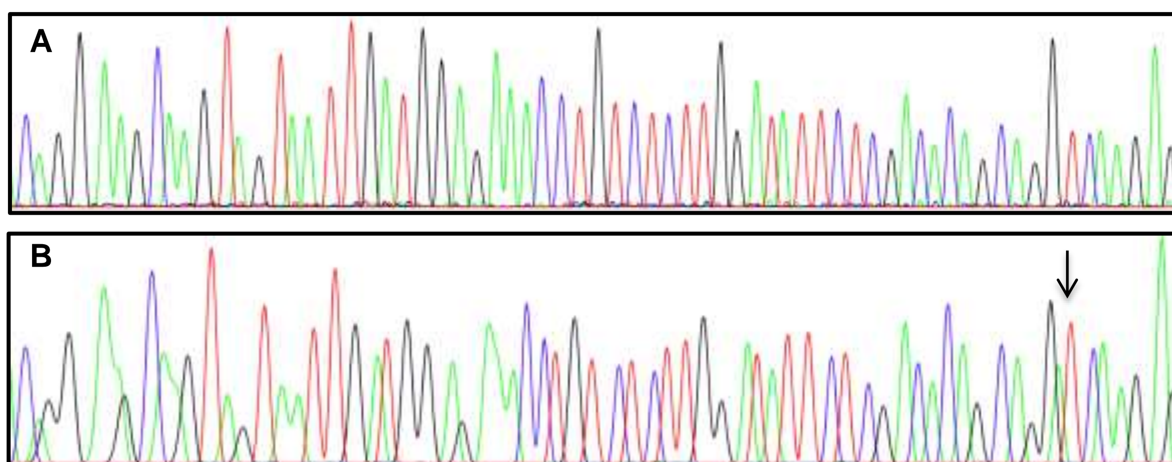


Figure 11. Electropherogram of *KRAS* exon 3 sequence, with A) a wild-type and B) a mutated sample.

Statistical analysis

Statistical analysis was performed using either Qui-square or Fisher's exact tests to assess statistical differences between the variants. Associations were considered statistically significant when $P \leq 0.05$. Statistical analysis was performed with the SPSS Statistics software package v.22.0.

Results

IV. RESULTS

DNA from a total of 241 *KRAS* exon 2 wild-type mCRC samples were screened in parallel for mutations in exons 3 and 4 of *KRAS* and exons 2, 3 and 4 of *NRAS* by HRM and automated sequencing. Automated sequencing of the HRM products confirmed the presence of 46 mutations (19.1%) in *KRAS* exons 3/4 or *NRAS* exons 2/3/4, with the remaining 80.9% (195/241) being wild-type for all regions studied. All mutations were found in heterozygosity and as a single mutation.

Table 5. Mutational status of the 241 mCRC samples analyzed.

Samples	
Mutational Status	**Frequencies**
Mutant	46
Wild-type	195
Total	**241**

Mutational Type and Distribution

Overall, 12.4% (30/241) of the cases presented a mutation in *KRAS* and 6.6% (16/241) were *NRAS* mutated. The mutational distribution of the 46 positive cases was as follows: 65.2% (30/46) in *KRAS*, with 28.3% (13/46) in *KRAS* exon 3 and 37.0% (17/46) in *KRAS* exon 4, and 34.8% (16/46) in *NRAS*, with 17.4% (8/46) in *NRAS* exon 2 and 17.4% (8/46)

in *NRAS* exon 3 (Figure 12). No mutations were found in exon 4 of *NRAS*. The individual mutations found in each gene are presented in Tables 6 and 7. Eleven different mutations were found in *KRAS* and seven different mutations were detected in *NRAS*. In all but two cases the mutations were missense, whereas the remaining two cases had an in frame duplication and an in frame deletion in *KRAS* exon 3.

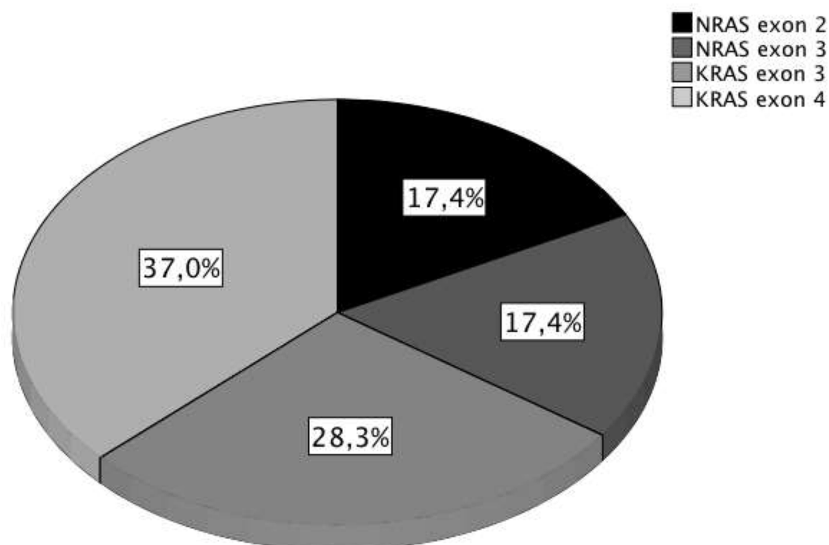


Figure 12. Distribution (%) of the 46 mutations detected in all analyzed exons in mCRC samples.

Table 6. ***KRAS*** **mutations identified after automated sequencing.**

KRAS				
Case	**Exon**	**Mutation**		**Nr.**
73	3	c.151_195dup	p.Cys51_Ser65dup	1
5	3	c.176_178del	p.Asp59del	1
216	3	c.175G>A	p.Ala59Thr	1
175	3	c.179G>A	p.Gly60Asp	1
68, 87, 138, 141, 192	3	c.182A>T	p.Gln61Leu	5
39, 84, 209	3	c.183A>C	p.Gln61His	3
224	3	c.183A>T	p.Gln61His	1
78, 93, 213	4	c.351A>T	p.Lys117Asn	3
165	4	c.351A>C	p.Lys117Asn	1
19, 30, 47, 49, 108, 119, 120, 131, 173, 200, 235	4	c.436G>A	p.Ala146Thr	11
149, 164	4	c.437C>T	p.Ala146Val	2
			Total	**30**

Table 7. *NRAS* mutations identified after automated sequencing

NRAS Case	Exon	Mutation		Nr.
31, 88	2	c.34G>T	p.Gly12Cys	2
40, 64, 118, 220, 228	2	c.35G>A	p.Gly12Asp	5
139	2	c.37G>C	p.Gly13Arg	1
13, 26, 227	3	c.181C>A	p.Gln61Lys	3
38, 111	3	c.182A>G	p.Gln61Arg	2
124	3	c.182A>T	p.Gln61Leu	1
4, 52	3	c.183A>T	p.Gln61His	2
			Total	**16**

Novel Mutations

Of the 11 different *KRAS* mutations and seven different *NRAS* mutations identified in this study, the mutation c.183A>T, p.Gln61His, is novel (Figure 13) and the remaining 17 mutations have previously been reported in the COSMIC database (COSMIC) or in the literature.

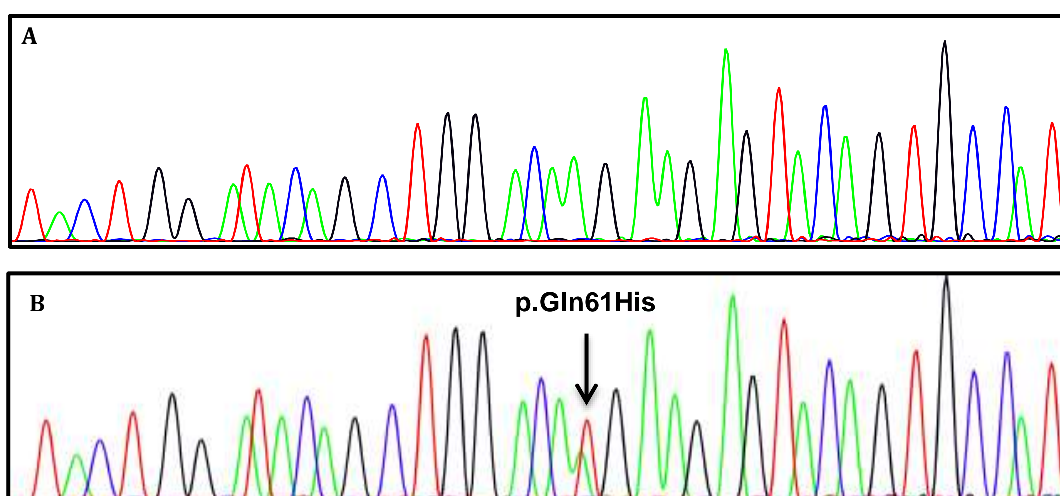


Figure 13. Electropherograms of the mutation found in *NRAS* exon 3 that was not previously described, with A) wild-type and B) mutant sample.

Clinicopathological Associations

The establishment of associations between the tumor genetic alterations and clinicopathological features was possible in 215 out of 241 cases.

Mutation frequencies in this subgroup are described below (Table 8/Figure 14). Qui-square or Fisher's exact tests (each one used when appropriate) were done to assess differences between *KRAS* and *NRAS* mutation distribution and the following variables: sex, age and stage at diagnosis, and primary tumor site.

Table 8. Mutational status in the subgroup of cases with available clinical data.

Samples	
Mutational Status	**Frequencies**
KRAS mutant	28
NRAS mutant	12
Wild-type	175
Total	**215**

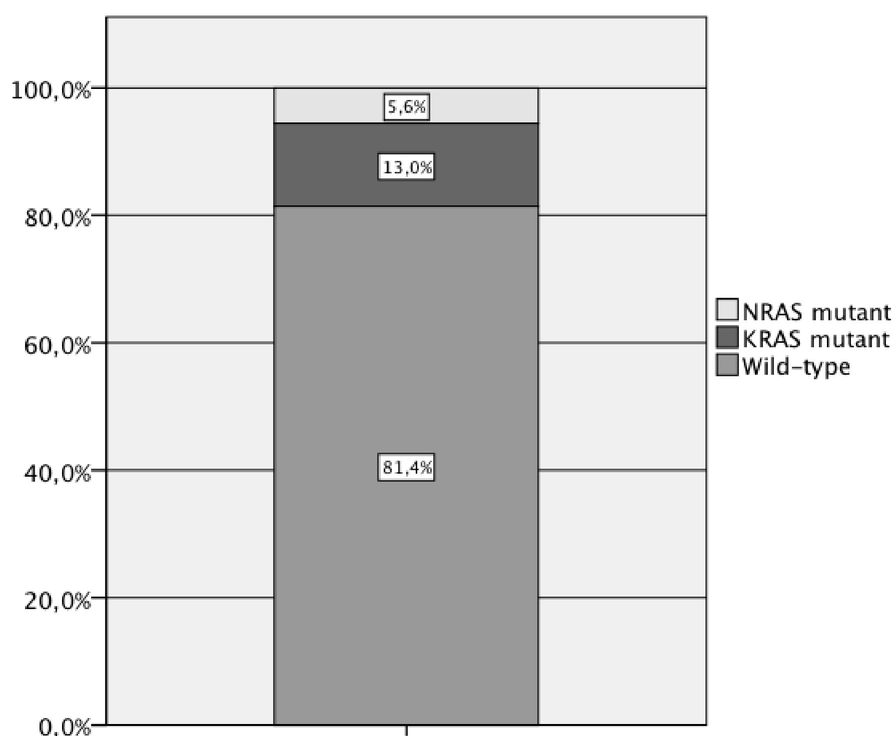
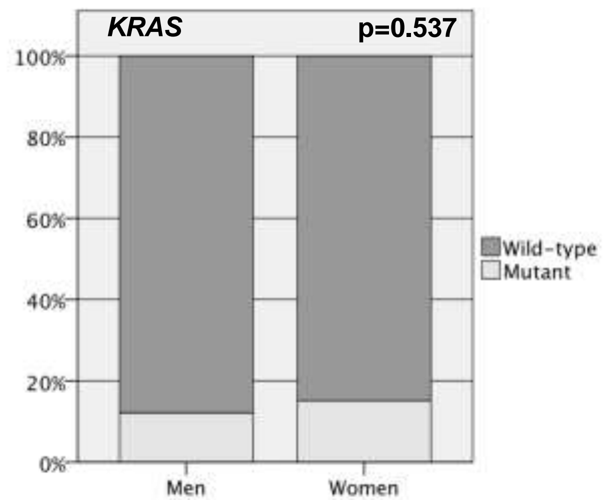


Figure 14. Mutational status (%) in the subgroup of cases with available clinical data.

Table 9. Distribution of *KRAS* and *NRAS* mutations according to patient sex.

Sex	*KRAS*		Total
	Wild-Type	Mutant	
Men	131	18	149
Women	56	10	66
Total	187	28	**215**



Sex	*NRAS*		Total
	Wild-Type	Mutant	
Men	142	7	149
Women	61	5	66
Total	203	12	**215**

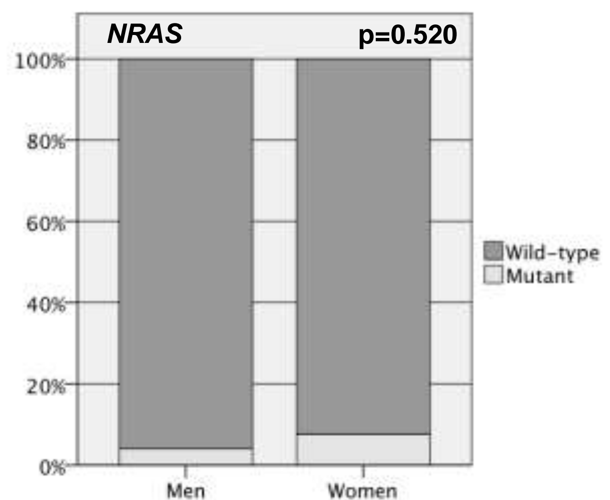
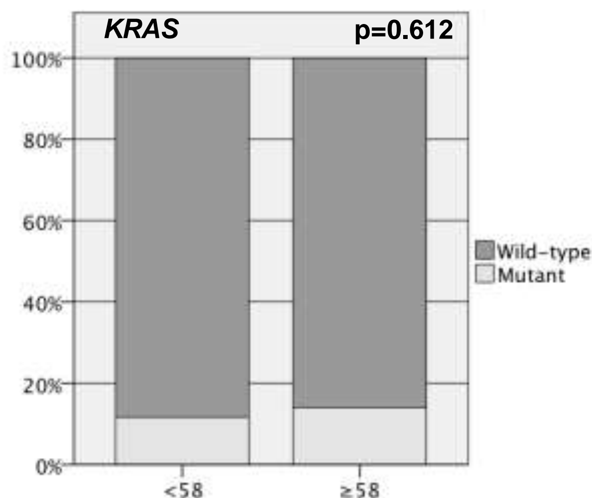


Figure 15. Distribution of *KRAS* and *NRAS* mutations according to patient sex.

No differences were found regarding *KRAS* or *NRAS* mutation distribution by patient gender: 12.1% in men *vs.* 15.2% in women (p=0.537) for *KRAS* and 4.7% in men *vs.* 7.6% in women (p=0.520) for *NRAS*.

Table 10. Distribution of *KRAS* and *NRAS* mutations according to patient age at diagnosis.

Age at diagnosis	*KRAS*		Total
	Wild-Type	Mutant	
<58	83	11	94
≥58	104	17	121
Total	187	28	**215**



Age at diagnosis	*NRAS*		Total
	Wild-Type	Mutant	
<58	91	3	94
≥58	112	9	121
Total	203	12	**215**

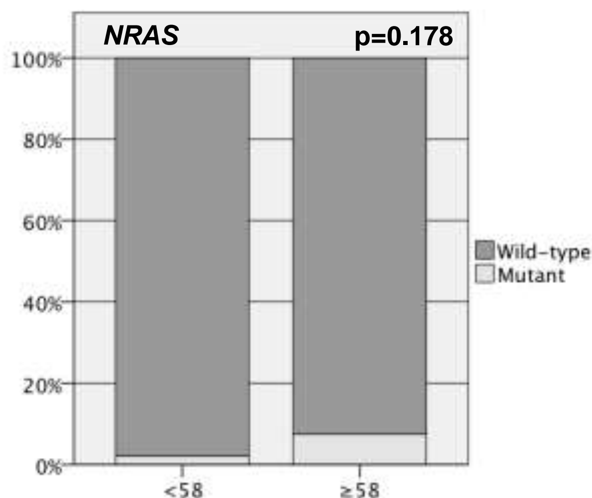


Figure 16. Distribution of *KRAS* and *NRAS* mutations according to patient age at diagnosis.

Age at diagnosis was divided into two groups (<58 and ≥58, with 58 being the average age at diagnosis) for statistical purposes. No statistically significant differences were found in *KRAS* (p=0.612) or *NRAS* (p=0.178) mutation distribution according to age at diagnosis.

Table 11. Distribution of *KRAS* and *NRAS* mutations according to patient stage at diagnosis.

Stage at diagnosis	*KRAS*		Total
	Wild-Type	Mutant	
I+II	45	15	60
III+IV	142	13	155
Total	187	28	**215**

Stage at diagnosis	*NRAS*		Total
	Wild-Type	Mutant	
I+II	56	4	60
III+IV	148	8	155
Total	203	12	**215**

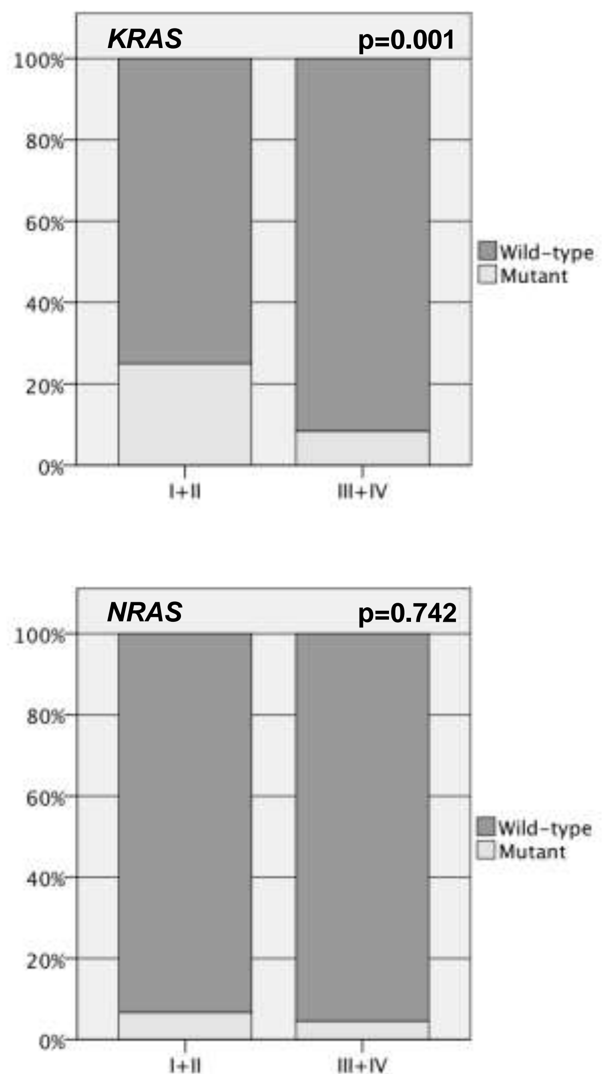
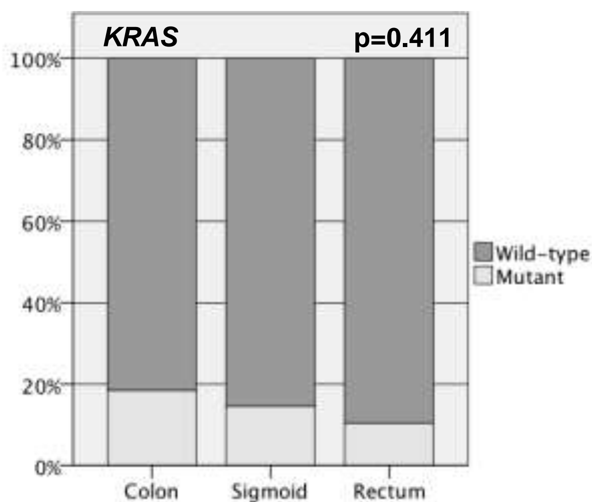


Figure 17. Distribution of *KRAS* and *NRAS* mutations according to patient stage at diagnosis.

KRAS mutations were more frequent (p=0.001) in earlier stages of diagnosis than in later ones (25.0% *vs.* 8.4%). However, *NRAS* mutations do not follow the same tendency, since no statistical differences were found between the two groups of stages (6.7% *vs.* 5.2 %; p=0.742).

Table 12. Distribution of *KRAS* and *NRAS* mutations according to primary tumor site.

Tumor site	*KRAS*		Total
	Wild-Type	Mutant	
Colon	31	7	38
Sigmoid	53	9	62
Rectum	103	12	99
Total	187	28	**215**



Tumor site	*NRAS*		Total
	Wild-Type	Mutant	
Colon	36	2	38
Sigmoid	60	2	62
Rectum	107	8	115
Total	203	12	**215**

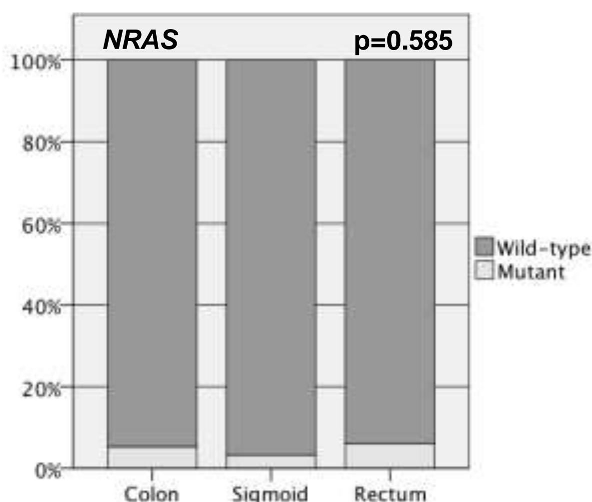


Figure 18. Distribution of *KRAS* and *NRAS* mutations according to primary tumor site.

There were relatively few tumors in the ascending, transverse and descending colon. For the purpose of this statistical analysis, the first three were grouped together as colon tumors. However, no statistical differences were found regarding *KRAS* (p=0.411) or *NRAS* (p=0.585) mutation distribution by primary tumor site.

DISCUSSION

V. DISCUSSION

Important progress has been made in recent years regarding treatment of CRC, with the introduction of new therapies that improve patient survival even after metastasis development. The administration of anti-EGFR to mCRC patients negative for *KRAS* exon 2 (codons 12/13) mutations improved considerably the outcome of those patients. These mutations occur in about 40% of mCRC patients and were established as the first negative predictors of response to anti-EGFR therapy. However, only 40 to 60% of all patients *KRAS* exon 2 wild-type achieve an objective response to this therapy (De Roock *et al.*, 2008; Lievre *et al.*, 2008). Such findings suggest that alterations in other EGFR downstream effectors may also predict response and lead to a further improvement of patient selection.

Over the years, several studies analyzed the effect of *KRAS* mutations in response to anti-EGFR therapy, with the majority including only the mutational analysis of *KRAS* exon 2 (codons 12/13) (Amado *et al.*, 2008; Douillard *et al.*, 2010; Peeters *et al.*, 2010). However, recent studies show that less frequent mutations in *KRAS* exons 3 and 4 and mutations in *NRAS* exons 2, 3 and 4 are also associated with resistance to anti-EGFR therapy in mCRC (De Roock *et al.*, 2010; Douillard *et al.*, 2013; Peeters *et al.*, 2013; Ciardiello *et al.*, 2014). In fact, it was reported that patients with activating *RAS* mutations do not benefit from this therapy and may in fact be harmed by its administration (Douillard *et al.*, 2013; Ciardiello *et al.*, 2014).

In a consecutive series of 241 mCRC samples wild-type for *KRAS* codons 12 and 13, we searched for mutations in the less frequently mutated *KRAS* mutational hotspots in exon 3 (codons 59/61) and 4 (codons 117/146) and in exon 2 (codons 12/13), 3 (codons 59/61) and 4 (codons 117/146) of *NRAS*. These hotspots are located in the P-loop domain (exon 2), switch II (exon 3) and G4/G5 regions (exon 4) of the highly conserved G domain, which is a common structure among RAS proteins (Edkins *et al.*, 2006; Schubbert *et al.*, 2007). Initially, all samples were screened by HRM for mutations in *KRAS* and *NRAS*. Subsequently, automated DNA sequencing was performed in all HRM products, in order to identify the alterations associated with each of the mutant cases. HRM was used as a screening mutation method, instead of a regular PCR, since this technique is a very accurate, fast and sensitive method that allows the detection of a small fraction of mutated alleles in tumor samples (~5%), through the evaluation of the different melting patterns obtained from wild-type sequences *vs.* heterozygote variants (Krypuy *et al.*, 2006; Pinto *et al.*, 2011). Furthermore, sequencing of HRM products increases sensibility in mutation detection from 85% to 98% (Pinto *et al.*, 2011). All HRM products were sequenced due to the fact that we obtained different rates of amplification among our samples and because of the use of big amplicons, such as those of *KRAS* and *NRAS* exon 4, which might decrease the sensitivity of mutation detection through HRM (Krypuy *et al.*, 2006; Do *et al.*, 2008).

The frequency of RAS mutations in this series (46/241 – 19.1%) is similar to that reported in recent studies with *KRAS* exon 2 wild-type mCRC, which ranges from approximately 15 to 20% (Vaughn *et al.*, 2011; Douillard *et al.*, 2013; Negru *et al.*, 2014; Sorich *et al.*, 2014). The mutational distribution of the 46 mutations is the following: 12.4% (30/241) were found in *KRAS*, 5.4% (13/241) and 7.1% (17/241) in exons 3 and 4, respectively; and 6.6% (16/241) were found in *NRAS*, 3.3% (8/241) in exon 2 and 3.3% (8/241) in exon 3. Although this mutational distribution slightly differs from that reported by Negru and collaborators (1.9% and 3.8% for *KRAS* exons 3 [codons 59/61] and 4 [codons 117/146], and 7.8% and 1.9% for *NRAS* exons 2 [codons 12/13] and 3 [codons 59/61], respectively), it is very similar to that reported by Sorich and collaborators in a recent systematic review and meta-analysis of nine randomized controlled trials compromising a total of 5948 patients (4.3% and 6.7% for *KRAS* exons 3 [codons 59/61] and 4 [codons 117/146], and 3.8% and 4.8% for *NRAS* exons 2 [codons 12/13] and 3 [codons 59/61], respectively) (Negru *et al.*, 2014; Sorich *et al.*, 2014). We did not detect mutations in *NRAS* exon 4 (codons 117/146), which seems to be a rare event in CRC, as indicated by the reported frequency ranging from 0.2 to 1% (Douillard *et al.*, 2013; Negru *et al.*, 2014; Sorich *et al.*, 2014).

Aberrant RAS function found in cancer cells is typically associated with mutations in codons 12, 13 or 61, since these codons, located in the P-loop (codons 12 and 13) and in the switch region II (codon 61), play an important role in the maintenance of the GTP-GDP transition state. Mutations in these sites impair GTP hydrolysis and lead to the oncogenic activation of the protein (Scheffzek *et al.*, 1997; Schubbert *et al.*, 2007; Prior *et al.*, 2012). Furthermore, it was demonstrated that the substitution of the Gln61 residue by other amino acids abolished GAP-dependent GTPase activation, leading to a constitutive activation of the RAS protein. This indicates that this amino acid is essential for GAP connection specificity to RAS GTPases (Nur & Maruta, 1992). In the present series only the *NRAS* gene was analyzed for codons 12 and 13 and the eight mutations detected resulted in three amino acid substitutions: p.Gly12Cys, p.Gly12Asp and Gly13Arg. Although the most frequent Gly12 mutant in our series was the Gly12Asp (5/8; 62.5%), its oncogenic potential is smaller than that of Gly12Val or Gly12Arg mutants (Schubbert *et al.*, 2007; Prior *et al.*, 2012), which we did not find.

Codon 61 was analyzed in both *KRAS* and *NRAS* genes and nine mutations were found in *KRAS* and eight in *NRAS*, representing four different amino acid substitutions: p.Gln61Lys, p.Gln61Arg, p.Gln61Leu and p.Gln61His. One third (2/6) of all p.Gln61His mutants were found in *NRAS* and, according to the literature and the COSMIC database, this alteration has not previously been reported in this gene in CRC. Although there are no data concerning its oncogenic proprieties, the fact that it is located in Gln61 might be an indicator of its role in RAS activation. Just as for Gly12, Gln61 mutants have various transformation efficiencies that vary from 10 to 1000-fold. One of the highest transformation efficiencies is seen with the p.Gln61Leu mutant (Buhrman *et al.*, 2007), which is also the most frequent Gln61 mutant in our series (7/17; 41.2%). However, in an analysis made by Vaughn and collaborators, p.Gln61Leu was found in only 17.1% (6/35) of *KRAS* and *NRAS* codon 61 mutations (Vaughn *et al.*, 2011). We also observed that, despite their high degree of homology, the frequency of mutations in these three hotspots differs between these two RAS proteins. In *KRAS*, mutations in codons 12 and 13 are generally more frequent than in codon 61, however in our series mutations in *NRAS* were more frequent in codon 61 than in codons 12 and 13 (50% *vs.* 43.75% *vs.* 6.25%, respectively), which is in accordance with the literature (Fernandez-Medarde & Santos, 2011; Prior *et al.*, 2012).

Due to a persistent bias in mutation screening over the years, the role of mutations in codons such as 59, 117 or 146 has been overlooked. Mutational analysis of these three codons was performed in our series, and mutations were found in all of them. Ala59 mutants found in our series were all located in *KRAS* and included one point mutation (p.Ala59Thr), one in frame deletion (p.Ala59del) and one large in-frame duplication

(p.Cys51_Ser65dup). There are no sufficient data to understand how these alterations might influence RAS protein structure and function, but the fact that this codon is located in the switch region II, the same as codon 61, indicates that mutations in this codon might also influence the transition complex during GTP hydrolysis (Macaluso *et al.*, 2002).

On the other hand, mutations in codons 117 and 146, which are involved with guanine base interaction, are known to increase the GDP to GTP exchange rate without affecting the GTPase activity (Edkins *et al.*, 2006). In fact, *in vivo* expression of both mutants resulted in elevated RAS-GTP expression compared with wild-type RAS, although lower than the one observed with *KRAS* codons 12 and 13 alleles (Janakiraman *et al.*, 2010). In our series, mutations in these codons were also found only in *KRAS*, with four mutations in codon 117 and thirteen in codon 146 (23.5% and 76.5%, respectively). These mutations originated three different mutants, Lys117Asn, Ala146Val and Ala146Thr, with the latter being the most frequent mutant out of the three (11/17; 64.7%), something that is consistent with the findings in other publications (Janakiraman *et al.*, 2010; Vaughn *et al.*, 2011).

Besides those mentioned above, we found one more mutation in *KRAS* exon 3, previously described by Molinari and collaborators (Molinari *et al.*, 2011). This mutation, p.Gly60Asp, has no functional studies that can confirm its role as an activating mutation. However, this residue is a conserved amino acid in the superfamily of GTPases and is known to interact with ϒ-phosphate of GTP, which is consistent with the hypothesis that a mutation in this codon might be oncogenic (Bourne *et al.*, 1991; Guedes *et al.*, 2013).

It is also important to mention the mutually exclusive distribution of mutations among *KRAS* and *NRAS* exons obtained in our series, since we only found single mutations in our pool of cases. This information suggests that alterations in these genes confer overlapping downstream effects due to functional redundancy, which is consistent with findings across the literature (De Roock *et al.*, 2010; Janakiraman *et al.*, 2010; Douillard *et al.*, 2013).

In the 215 cases with available clinical data, we tested for association between RAS mutations and clinicopathological features, such as gender, age and stage at diagnosis, and primary tumor site. Interestingly, an association was found between *KRAS* mutations ($p=0.001$) and earlier tumor stages at diagnosis, an association that was previously described (Fernandez-Medarde & Santos, 2011). No other statistically significant associations were found, but this might be due to the relatively small sample size and these findings should therefore be confirmed in larger series.

Although it had been already suggested in the past (De Roock *et al.*, 2010), the importance of RAS mutations, besides those in codons 12 and 13 of *KRAS*, as predictors of resistance to anti-EGFR has only recently been established. Douillard and collaborators

published recently the results of the PRIME trial, which assessed the efficacy and safety of adding panitumumab to FOLFOX4 in RAS mutated patients (Douillard *et al.*, 2013). Of the 1183 patients who underwent randomization, 108 patients (17%; 108/620) without *KRAS* mutations in exon 2 had mutations in other RAS exons. In this subgroup of patients, the analysis showed that PFS and overall survival (OS) observed were shorter in the panitumumab-FOLFOX4 group than in the FOLFOX4-alone group (7.3 *vs.* 8.0 months, p=0.33; 17.1 *vs.* 18.3 months, p=0.31). Although the difference was not significant, these outcomes were consistent with those found for the subgroup of patients with *KRAS* mutations in exon 2. Moreover, patients without RAS mutations in the panitumumab-FOLFOX4 group were associated with a significant improvement in progression free survival (10.1 *vs.* 7.9 moths, p=0.004) and overall survival (26.0 *vs.* 20.2, p=0.04), when compared with FOLFOX-alone.

Similar results, concerning the addition of cetuximab to FOLFIRI in the treatment of mCRC patients, were reported by Ciardiello and collaborators in the latest results from the CRYSTAL trial (Ciardiello *et al.*, 2014). 1198 randomized and treated patients were evaluated in this trial, and 14.7% (63/430) of those considered wild-type for *KRAS* codons 12 and 13 tumors had other RAS mutations. The differences reported for PFS and OS in this subgroup, between the cetuximab-FOLFIRI and the FOLFIRI-alone groups, were not statistically significant (7.2 vs. 6.9 months, p=0.56; 18.2 vs. 20.7 months, p=0.50). However, when compared with the RAS wild-type subgroup results (11.4 vs. 8.4 months, p=0.0002; 28.4 vs. 20.2 months, p=0.0024) it is possible to conclude that the addition of cetuximab to FOLFIRI has no benefit for patients with RAS mutations. All these findings suggest that RAS activating mutations, in addition to *KRAS* exon 2 mutations, predict lack of response in patients who received anti-EGFR therapy (cetuximab or panitumumab).

Due to the absence of information, at the time of writing, on the outcome of the RAS mutated patients treated with cetuximab/panitumumab, we could not evaluate the role of RAS mutations, as predictive biomarkers of treatment response, in this series of patients. However, considering the results obtained in our mutational analysis of 241 cases and the findings by Douillard and collaborators (Douillard *et al.*, 2013) and Ciardiello and collaborators (Ciardiello *et al.*, 2014), we can expect that about one-fifth of patients considered wild-type for *KRAS* exon 2 are unlikely to benefit from anti-EGFR therapy due to the presence of other RAS mutations.

CONCLUSIONS

VI. Conclusions

Taking into account the results obtained in this study, we can conclude that:

I) HRM followed by automated Sanger sequencing of *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4 allows the detection of other RAS mutations in about one-fifth of 241 Portuguese mCRC patients wild-type for KRAS exon 2;

II) The 46 additional RAS mutations found are mutually exclusive and have the following distribution:

a. 5.4% in *KRAS* exon 3;
b. 7.1% in *KRAS* exon 4;
c. 3.3% in *NRAS* exon 2;
d. 3.3% in *NRAS* exon 3;

III) Eleven and seven different mutations were found in *KRAS* and *NRAS*, respectively, with a novel *NRAS* exon 3 mutation being found in two cases;

IV) In this setting, a statistically significant association was found between *KRAS* exon 3/4 mutations and early tumor stage at diagnosis.

FUTURE PERSPECTIVES

VII. FUTURE PERSPECTIVES

The results obtained in this work show that the overall frequency and type of mutations found in *KRAS* (exons 3 and 4) and *NRAS* (exons 2, 3 and 4) in Portuguese mCRC patients are in accordance with those previously reported in literature in other populations and may help to distinguish patients who are most likely to benefit from anti-EGFR therapy. However, further studies are still necessary to determine the full therapeutic implications of the mutations found in our series, including *in vitro* and *in vivo* tests to evaluate the oncogenic potential of the novel *NRAS* mutation here described.

It will be important to analyze all available clinical data of each mutated patient in order to identify those who were treated with cetuximab or panitumumab and to find out which were the therapy responses. The comparisons of these data with those of RAS wild-type patients treated with the same drugs will eventually allow us confirm their importance as negative predictors of response to anti-EGFR therapy.

Finally, mutational analysis of other potential predictive biomarkers of response, such as *BRAF* and *PIK3CA*, might contribute to further improve patient selection for effective anti-EGFR therapy in the future.

REFERENCES

VIII. REFERENCES

Adelstein, B. A., Dobbins, T. A., Harris, C. A., Marschner, I. C., & Ward, R. L. (2011). A systematic review and meta-analysis of KRAS status as the determinant of response to anti-EGFR antibodies and the impact of partner chemotherapy in metastatic colorectal cancer. *Eur J Cancer, 47*(9), 1343-1354.

Al-Sohaily, S., Biankin, A., Leong, R., Kohonen-Corish, M., & Warusavitarne, J. (2012). Molecular pathways in colorectal cancer. *J Gastroenterol Hepatol, 27*(9), 1423-1431.

Amado, R. G., Wolf, M., Peeters, M., Van Cutsem, E., Siena, S., Freeman, D. J., Juan, T., Sikorski, R., Suggs, S., Radinsky, R., Patterson, S. D., & Chang, D. D. (2008). Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol, 26*(10), 1626-1634.

Amersi, F., Agustin, M., & Ko, C. Y. (2005). Colorectal cancer: epidemiology, risk factors, and health services. *Clin Colon Rectal Surg, 18*(3), 133-140.

Bogaert, J., & Prenen, H. (2014). Molecular genetics of colorectal cancer. *Ann Gastroenterol, 27*(1), 9-14.

Bokemeyer, C., Bondarenko, I., Hartmann, J. T., de Braud, F., Schuch, G., Zubel, A., Celik, I., Schlichting, M., & Koralewski, P. (2011). Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. *Ann Oncol, 22*(7), 1535-1546.

Bokemeyer, C., Bondarenko, I., Makhson, A., Hartmann, J. T., Aparicio, J., de Braud, F., Donea, S., Ludwig, H., Schuch, G., Stroh, C., Loos, A. H., Zubel, A., & Koralewski, P. (2009). Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol, 27*(5), 663-671.

Bourne, H. R., Sanders, D. A., & McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature, 349*(6305), 117-127.

Buhrman, G., Wink, G., & Mattos, C. (2007). Transformation efficiency of RasQ61 mutants linked to structural features of the switch regions in the presence of Raf. *Structure, 15*(12), 1618-1629.

Cassidy, J., Saltz, L., Twelves, C., Van Cutsem, E., Hoff, P., Kang, Y., Saini, J. P., Gilberg, F., & Cunningham, D. (2011). Efficacy of capecitabine versus 5-fluorouracil in colorectal and gastric cancers: a meta-analysis of individual data from 6171 patients. *Ann Oncol, 22*(12), 2604-2609.

Centelles, J. J. (2012). General aspects of colorectal cancer. *ISRN Oncol, 2012*, 139268.

Chan, A. T., & Giovannucci, E. L. (2010). Primary prevention of colorectal cancer. *Gastroenterology, 138*(6), 2029-2043.e2010.

Chung, K. Y., Shia, J., Kemeny, N. E., Shah, M., Schwartz, G. K., Tse, A., Hamilton, A., Pan, D., Schrag, D., Schwartz, L., Klimstra, D. S., Fridman, D., Kelsen, D. P., & Saltz, L. B. (2005). Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol, 23*(9), 1803-1810.

Ciardiello, F., Lenz, H. J., Kohne, C. H., Heinemann, V., Tejpar, S., Melezinek, I., Beier, F., Stroh, C., & Van Cutsem, E. (2014). Treatment outcome according to tumor RAS mutation status in CRYSTAL study patients with metastatic colorectal cancer (mCRC) randomized to FOLFIRI with/without cetuximab. *J Clin Oncol, 32:5s*(abstr 3506).

Ciardiello, F., & Tortora, G. (2008). EGFR antagonists in cancer treatment. *N Engl J Med, 358*(11), 1160-1174.

Colussi, D., Brandi, G., Bazzoli, F., & Ricciardiello, L. (2013). Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. *Int J Mol Sci, 14*(8), 16365-16385.

COSMIC. COSMIC: The catalogue of somatic mutations in cancer.

Cummings, L. C., & Cooper, G. S. (2011). Colorectal cancer screening: update for 2011. *Semin Oncol, 38*(4), 483-489.

De Roock, W., Claes, B., Bernasconi, D., De Schutter, J., Biesmans, B., Fountzilas, G., Kalogeras, K. T., Kotoula, V., Papamichael, D., Laurent-Puig, P., Penault-Llorca, F., Rougier, P., Vincenzi, B., Santini, D., Tonini, G., Cappuzzo, F., Frattini, M., Molinari, F., Saletti, P., De Dosso, S., Martini, M., Bardelli, A., Siena, S., Sartore-Bianchi, A., Tabernero, J., Macarulla, T., Di Fiore, F., Gangloff, A. O., Ciardiello, F., Pfeiffer, P., Qvortrup, C., Hansen, T. P., Van Cutsem, E., Piessevaux, H., Lambrechts, D., Delorenzi, M., & Tejpar, S. (2010). Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol, 11*(8), 753-762.

De Roock, W., Piessevaux, H., De Schutter, J., Janssens, M., De Hertogh, G., Personeni, N., Biesmans, B., Van Laethem, J. L., Peeters, M., Humblet, Y., Van Cutsem, E., & Tejpar, S. (2008). KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol, 19*(3), 508-515.

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene, 26*(22), 3279-3290.

Do, H., Krypuy, M., Mitchell, P. L., Fox, S. B., & Dobrovic, A. (2008). High resolution melting analysis for rapid and sensitive EGFR and KRAS mutation detection in formalin fixed paraffin embedded biopsies. *BMC Cancer, 8*, 142.

Douillard, J. Y., Oliner, K. S., Siena, S., Tabernero, J., Burkes, R., Barugel, M., Humblet, Y., Bodoky, G., Cunningham, D., Jassem, J., Rivera, F., Kocakova, I., Ruff, P., Blasinska-Morawiec, M., Smakal, M., Canon, J. L., Rother, M., Williams, R., Rong, A., Wiezorek, J., Sidhu, R., & Patterson, S. D. (2013). Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med, 369*(11), 1023-1034.

Douillard, J. Y., Siena, S., Cassidy, J., Tabernero, J., Burkes, R., Barugel, M., Humblet, Y., Bodoky, G., Cunningham, D., Jassem, J., Rivera, F., Kocakova, I., Ruff, P., Blasinska-Morawiec, M., Smakal, M., Canon, J. L., Rother, M., Oliner, K. S., Wolf, M., & Gansert, J. (2010). 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, 28*(31), 4697-4705.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer, 3*(1), 11-22.

Edge, S., Byrd, D., & Compton, C. (2010). AJCC Cancer Staging Manual, 7th edition. *Springer*.

Edkins, S., O'Meara, S., Parker, A., Stevens, C., Reis, M., Jones, S., Greenman, C., Davies, H., Dalgliesh, G., Forbes, S., Hunter, C., Smith, R., Stephens, P., Goldstraw, P., Nicholson, A., Chan, T. L., Velculescu, V. E., Yuen, S. T., Leung, S. Y., Stratton, M. R., & Futreal, P. A. (2006). Recurrent KRAS codon 146 mutations in human colorectal cancer. *Cancer Biol Ther, 5*(8), 928-932.

Edwards, M. S., Chadda, S. D., Zhao, Z., Barber, B. L., & Sykes, D. P. (2012). A systematic review of treatment guidelines for metastatic colorectal cancer. *Colorectal Dis, 14*(2), e31-47.

El Zouhairi, M., Charabaty, A., & Pishvaian, M. J. (2011). Molecularly targeted therapy for metastatic colon cancer: proven treatments and promising new agents. *Gastrointest Cancer Res, 4*(1), 15-21.

Ellis, L. M. (2006). Mechanisms of action of bevacizumab as a component of therapy for metastatic colorectal cancer. *Semin Oncol, 33*(5 Suppl 10), S1-7.

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell, 61*(5), 759-767.

Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W., Comber, H., Forman, D., & Bray, F. (2013). Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer, 49*(6), 1374-1403.

Fernandez-Medarde, A., & Santos, E. (2011). Ras in cancer and developmental diseases. *Genes Cancer, 2*(3), 344-358.

Gingras, D., & Beliveau, R. (2011). Colorectal cancer prevention through dietary and lifestyle modifications. *Cancer Microenviron, 4*(2), 133-139.

Guedes, J. G., Veiga, I., Rocha, P., Pinto, P., Pinto, C., Pinheiro, M., Peixoto, A., Fragoso, M., Raimundo, A., Ferreira, P., Machado, M., Sousa, N., Lopes, P., Araujo, A., Macedo, J., Alves, F., Coutinho, C., Henrique, R., Santos, L. L., & Teixeira, M. R. (2013). High resolution melting analysis of KRAS, BRAF and PIK3CA in KRAS exon 2 wild-type metastatic colorectal cancer. *BMC Cancer, 13*, 169.

Hagan, S., Orr, M. C., & Doyle, B. (2013). Targeted therapies in colorectal cancer-an integrative view by PPPM. *Epma j, 4*(1), 3.

Haggar, F. A., & Boushey, R. P. (2009). Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clin Colon Rectal Surg, 22*(4), 191-197.

Hecht, J. R., Mitchell, E., Chidiac, T., Scroggin, C., Hagenstad, C., Spigel, D., Marshall, J., Cohn, A., McCollum, D., Stella, P., Deeter, R., Shahin, S., & Amado, R. G. (2009). A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. *J Clin Oncol, 27*(5), 672-680.

Hicklin, D. J., & Ellis, L. M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol, 23*(5), 1011-1027.

Hirsch, B. R., & Zafar, S. Y. (2011). Capecitabine in the management of colorectal cancer. *Cancer Manag Res, 3*, 79-89.

Huxley, R. R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L., & Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. *Int J Cancer, 125*(1), 171-180.

Hynes, N. E., & MacDonald, G. (2009). ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol, 21*(2), 177-184.

IARC. (2013). GLOBOCAN 2012: Cancer Incidence and Mortality Worldwide. IARC CancerBase No. 11 [Internet]. *Lyon, France: International Agency for Research on Cancer, Available from:* http://globocan.iarc.fr.

Ilyas, M., Straub, J., Tomlinson, I. P., & Bodmer, W. F. (1999). Genetic pathways in colorectal and other cancers. *Eur J Cancer, 35*(14), 1986-2002.

Janakiraman, M., Vakiani, E., Zeng, Z., Pratilas, C. A., Taylor, B. S., Chitale, D., Halilovic, E., Wilson, M., Huberman, K., Ricarte Filho, J. C., Persaud, Y., Levine, D. A., Fagin, J. A., Jhanwar, S. C., Mariadason, J. M., Lash, A., Ladanyi, M., Saltz, L. B., Heguy, A., Paty, P. B., & Solit, D. B. (2010). Genomic and biological characterization of exon 4 KRAS mutations in human cancer. *Cancer Res, 70*(14), 5901-5911.

Jasperson, K. W., Tuohy, T. M., Neklason, D. W., & Burt, R. W. (2010). Hereditary and familial colon cancer. *Gastroenterology, 138*(6), 2044-2058.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA Cancer J Clin, 61*(2), 69-90.

Kanthan, R., Senger, J. L., & Kanthan, S. C. (2012). Molecular events in primary and metastatic colorectal carcinoma: a review. *Patholog Res Int, 2012*, 597497.

Karapetis, C. S., Khambata-Ford, S., Jonker, D. J., O'Callaghan, C. J., Tu, D., Tebbutt, N. C., Simes, R. J., Chalchal, H., Shapiro, J. D., Robitaille, S., Price, T. J., Shepherd, L., Au, H. J., Langer, C., Moore, M. J., & Zalcberg, J. R. (2008). K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med, 359*(17), 1757-1765.

Karnoub, A. E., & Weinberg, R. A. (2008). Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol, 9*(7), 517-531.

Kerbel, R. S. (2008). Tumor angiogenesis. *N Engl J Med, 358*(19), 2039-2049.

Krypuy, M., Newnham, G. M., Thomas, D. M., Conron, M., & Dobrovic, A. (2006). High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer, 6*, 295.

Labianca, R., Nordlinger, B., Beretta, G. D., Brouquet, A., & Cervantes, A. (2010). Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up. *Ann Oncol, 21 Suppl 5*, v70-77.

Laurent-Puig, P., Cayre, A., Manceau, G., Buc, E., Bachet, J. B., Lecomte, T., Rougier, P., Lievre, A., Landi, B., Boige, V., Ducreux, M., Ychou, M., Bibeau, F., Bouche, O., Reid, J., Stone, S., & Penault-Llorca, F. (2009). Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol, 27*(35), 5924-5930.

Leggett, B., & Whitehall, V. (2010). Role of the serrated pathway in colorectal cancer pathogenesis. *Gastroenterology, 138*(6), 2088-2100.

Lentz, F., Tran, A., Rey, E., Pons, G., & Treluyer, J. M. (2005). Pharmacogenomics of fluorouracil, irinotecan, and oxaliplatin in hepatic metastases of colorectal cancer: clinical implications. *Am J Pharmacogenomics, 5*(1), 21-33.

Levin, B., Lieberman, D. A., McFarland, B., Smith, R. A., Brooks, D., Andrews, K. S., Dash, C., Giardiello, F. M., Glick, S., Levin, T. R., Pickhardt, P., Rex, D. K., Thorson, A., & Winawer, S. J. (2008). Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *CA Cancer J Clin, 58*(3), 130-160.

Lievre, A., Bachet, J. B., Boige, V., Cayre, A., Le Corre, D., Buc, E., Ychou, M., Bouche, O., Landi, B., Louvet, C., Andre, T., Bibeau, F., Diebold, M. D., Rougier, P., Ducreux, M., Tomasic, G., Emile, J. F., Penault-Llorca, F., & Laurent-Puig, P. (2008). KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol, 26*(3), 374-379.

Lynch, H. T., Lynch, J. F., & Attard, T. A. (2009). Diagnosis and management of hereditary colorectal cancer syndromes: Lynch syndrome as a model. *Cmaj, 181*(5), 273-280.

Macaluso, M., Russo, G., Cinti, C., Bazan, V., Gebbia, N., & Russo, A. (2002). Ras family genes: an interesting link between cell cycle and cancer. *J Cell Physiol, 192*(2), 125-130.

Markowitz, S. D., & Bertagnolli, M. M. (2009). Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med, 361*(25), 2449-2460.

Marshall, J. (2006). Clinical implications of the mechanism of epidermal growth factor receptor inhibitors. *Cancer, 107*(6), 1207-1218.

Maughan, T. S., Adams, R., Smith, C. G., Seymour, M. T., Wilson, R. H., Meade, A. M., Fisher, D., Madi, A., Cheadle, J., & Kaplan, R. S. (2010). Identification of potentially responsive subsets when cetuximab is added to oxaliplatin-fluoropyrimidine chemotherapy in first-line advanced colorectal cancer: mature results of the MRC COIN trial. *J Clin Oncol, 28:15s*(abstr 3502).

Molinari, F., Felicioni, L., Buscarino, M., De Dosso, S., Buttitta, F., Malatesta, S., Movilia, A., Luoni, M., Boldorini, R., Alabiso, O., Girlando, S., Soini, B., Spitale, A., Di Nicolantonio, F., Saletti, P., Crippa, S., Mazzucchelli, L., Marchetti, A., Bardelli, A., & Frattini, M. (2011). Increased detection sensitivity for KRAS mutations enhances the prediction of anti-EGFR monoclonal antibody resistance in metastatic colorectal cancer. *Clin Cancer Res, 17*(14), 4901-4914.

Moran, A., Ortega, P., de Juan, C., Fernandez-Marcelo, T., Frias, C., Sanchez-Pernaute, A., Torres, A. J., Diaz-Rubio, E., Iniesta, P., & Benito, M. (2010). Differential colorectal carcinogenesis: Molecular basis and clinical relevance. *World J Gastrointest Oncol, 2*(3), 151-158.

Moroni, M., Veronese, S., Benvenuti, S., Marrapese, G., Sartore-Bianchi, A., Di Nicolantonio, F., Gambacorta, M., Siena, S., & Bardelli, A. (2005). Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol, 6*(5), 279-286.

NCCN. (2013). NCCN Clinical Practice Guidelines in Oncology. *V.3*.

Negru, S., Papadopoulou, E., Apessos, A., Stanculeanu, D. L., Ciuleanu, E., Volovat, C., Croitoru, A., Kakolyris, S., Aravantinos, G., Ziras, N., Athanasiadis, E., Touroutoglou, N., Pavlidis, N., Kalofonos, H. P., & Nasioulas, G. (2014). KRAS, NRAS and BRAF mutations in Greek and Romanian patients with colorectal cancer: a cohort study. *BMJ Open, 4*(5), e004652.

Noordhuis, P., Holwerda, U., Van der Wilt, C. L., Van Groeningen, C. J., Smid, K., Meijer, S., Pinedo, H. M., & Peters, G. J. (2004). 5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers. *Ann Oncol, 15*(7), 1025-1032.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., Carotenuto, A., De Feo, G., Caponigro, F., & Salomon, D. S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene, 366*(1), 2-16.

Nur, E. K. M. S., & Maruta, H. (1992). The role of Gln61 and Glu63 of Ras GTPases in their activation by NF1 and Ras GAP. *Mol Biol Cell, 3*(12), 1437-1442.

Peeters, M., Oliner, K. S., Parker, A., Siena, S., Van Cutsem, E., Huang, J., Humblet, Y., Van Laethem, J. L., Andre, T., Wiezorek, J., Reese, D., & Patterson, S. D. (2013). Massively parallel tumor multigene sequencing to evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer. *Clin Cancer Res, 19*(7), 1902-1912.

Peeters, M., Price, T. J., Cervantes, A., Sobrero, A. F., Ducreux, M., Hotko, Y., Andre, T., Chan, E., Lordick, F., Punt, C. J., Strickland, A. H., Wilson, G., Ciuleanu, T. E., Roman, L., Van Cutsem, E., Tzekova, V., Collins, S., Oliner, K. S., Rong, A., & Gansert, J. (2010). Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line

treatment in patients with metastatic colorectal cancer. *J Clin Oncol, 28*(31), 4706-4713.

Pino, M. S., & Chung, D. C. (2010). The chromosomal instability pathway in colon cancer. *Gastroenterology, 138*(6), 2059-2072.

Pinto, P., Rocha, P., Veiga, I., Guedes, J., Pinheiro, M., Peixoto, A., Pinto, C., Fragoso, M., Sanches, E., Araujo, A., Alves, F., Coutinho, C., Lopes, P., Henrique, R., & Teixeira, M. R. (2011). Comparison of methodologies for KRAS mutation detection in metastatic colorectal cancer. *Cancer Genet, 204*(8), 439-446.

Prior, I. A., Lewis, P. D., & Mattos, C. (2012). A comprehensive survey of Ras mutations in cancer. *Cancer Res, 72*(10), 2457-2467.

Roberts, P. J., & Der, C. J. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene, 26*(22), 3291-3310.

Rustgi, A. K. (2007). The genetics of hereditary colon cancer. *Genes Dev, 21*(20), 2525-2538.

Santarpia, L., Lippman, S. M., & El-Naggar, A. K. (2012). Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. *Expert Opin Ther Targets, 16*(1), 103-119.

Scaltriti, M., & Baselga, J. (2006). The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin Cancer Res, 12*(18), 5268-5272.

Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F., & Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science, 277*(5324), 333-338.

Schmoll, H. J., Van Cutsem, E., Stein, A., Valentini, V., Glimelius, B., Haustermans, K., Nordlinger, B., van de Velde, C. J., Balmana, J., Regula, J., Nagtegaal, I. D., Beets-Tan, R. G., Arnold, D., Ciardiello, F., Hoff, P., Kerr, D., Kohne, C. H., Labianca, R., Price, T., Scheithauer, W., Sobrero, A., Tabernero, J., Aderka, D., Barroso, S., Bodoky, G., Douillard, J. Y., El Ghazaly, H., Gallardo, J., Garin, A., Glynne-Jones, R., Jordan, K., Meshcheryakov, A., Papamichail, D., Pfeiffer, P., Souglakos, I., Turhal, S., & Cervantes, A. (2012). ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. *Ann Oncol, 23*(10), 2479-2516.

Schubbert, S., Shannon, K., & Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer, 7*(4), 295-308.

Seymour, M. T., Brown, S. R., Middleton, G., Maughan, T., Richman, S., Gwyther, S., Lowe, C., Seligmann, J. F., Wadsley, J., Maisey, N., Chau, I., Hill, M., Dawson, L., Falk, S., O'Callaghan, A., Benstead, K., Chambers, P., Oliver, A., Marshall, H., Napp, V., & Quirke, P. (2013). Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. *Lancet Oncol, 14*(8), 749-759.

Shia, J., Klimstra, D. S., Bagci, P., Basturk, O., & Adsay, N. V. (2012). TNM staging of colorectal carcinoma: issues and caveats. *Semin Diagn Pathol, 29*(3), 142-153.

Sorich, M. J., Wiese, M. D., Rowland, A., Kichenadasse, G., McKinnon, R. A., & Karapetis, C. S. (2014). Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized controlled trials. *Ann Oncol*.

Spano, J. P., Fagard, R., Soria, J. C., Rixe, O., Khayat, D., & Milano, G. (2005). Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. *Ann Oncol, 16*(2), 189-194.

Tol, J., Koopman, M., Cats, A., Rodenburg, C. J., Creemers, G. J., Schrama, J. G., Erdkamp, F. L., Vos, A. H., van Groeningen, C. J., Sinnige, H. A., Richel, D. J., Voest, E. E., Dijkstra, J. R., Vink-Borger, M. E., Antonini, N. F., Mol, L., van Krieken, J. H., Dalesio, O., & Punt, C. J. (2009). Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *N Engl J Med, 360*(6), 563-572.

Tveit, K., Guren, T., Glimelius, B., Pfeiffer, P., Sorbye, H., Pyrhonen, S., Kure, E., Ikdahl, T., Skovlund, E., & Christoffersen, T. (2010). Randomized phase III study of 5-

fluorouracil/folinate/oxaliplatin given continuously or intermittently with or without cetuximab, as first-line treatment of metastatic colorectal cancer: The Nordic VII study (NCT00145314), by the Nordic Colorectal Cancer Biomodulation Group. *Ann Oncol, 21 Suppl 8*(abstr LBA20).

Van Cutsem, E., Kohne, C. H., Hitre, E., Zaluski, J., Chang Chien, C. R., Makhson, A., D'Haens, G., Pinter, T., Lim, R., Bodoky, G., Roh, J. K., Folprecht, G., Ruff, P., Stroh, C., Tejpar, S., Schlichting, M., Nippgen, J., & Rougier, P. (2009). Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med, 360*(14), 1408-1417.

Van Cutsem, E., Lang, I., Folprecht, G., Nowacki, M., Cascinu, S., Shchepotin, I., Maurel, J., Cunningham, D., Celik, I., & Kohne, C. (2010). Cetuximab plus FOLFIRI in the treatment of metastatic colorectal cancer: The influence of KRAS and BRAF biomarkers on outcome: Updated data from the CRYSTAL trial. *American Society of Clinical Oncology (ASCO): Gastrointestinal Cancers Symposium*(abstr 281).

Van Cutsem, E., Nordlinger, B., & Cervantes, A. (2010). Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. *Ann Oncol, 21 Suppl 5*, v93-97.

Vaughn, C. P., Zobell, S. D., Furtado, L. V., Baker, C. L., & Samowitz, W. S. (2011). Frequency of KRAS, BRAF, and NRAS mutations in colorectal cancer. *Genes Chromosomes Cancer, 50*(5), 307-312.

Worthley, D. L., Whitehall, V. L., Spring, K. J., & Leggett, B. A. (2007). Colorectal carcinogenesis: road maps to cancer. *World J Gastroenterol, 13*(28), 3784-3791.

Yu, M., Trobridge, P., Wang, Y., Kanngurn, S., Morris, S. M., Knoblaugh, S., & Grady, W. M. (2014). Inactivation of TGF-beta signaling and loss of PTEN cooperate to induce colon cancer in vivo. *Oncogene, 33*(12), 1538-1547.