UNIVERSIDADE DO PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR

COMPARISON OF METHODOLOGIES FOR *KRAS* MUTATION DETECTION IN METASTATIC COLORECTAL CANCER

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Dissertação de Mestrado em Oncologia

Porto, 2009

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"Comparison of methodologies for KRAS mutation detection in metastatic colorectal cancer"

Dissertação de candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto e à Thomas Jefferson University.

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Categoria: Técnico Superior de Saúde

Afiliação: Instituto Português de Oncologia do Porto

Aknowledgements

To Prof. Doutor Manuel Teixeira, supervisor of this thesis, for the opportunity of doing research in oncology, for all the support during the development of this work and for being available all the time.

To Dra. Ana Peixoto, co-supervisor of this thesis, for all the knowledge, ideas and great support provided. Thank you for all the motivation given during this last year.

To Prof. Carlos Lopes, Director of the Master's in Oncology, for all the effort demonstrated.

To Liga Portuguesa contra o Cancro, for the financial support.

To all the colleagues in the Genetics Department, for receiving me so well and providing me a friendly work environment.

To Isabel and Patricia, for helping me throughout the development of this thesis.

To Catarina and Carla, for all the friendship, advice and support.

To Manuela, a special thanks for the constant support and encouragement. And for all the fun moments spent together.

To my family, for everything they taught me and for being present in all moments.

To Andreia, for the love, friendship, affection and patience. None of this would have been possible without you.

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Abbreviations

BSC	Best supportive care
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration
FdUMP	Fluorodeoxyuridine monophosphate
FFPE	Formalin-fixed paraffin-embedded
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H&E	Hematoxylin and eosin
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HRM	High Resolution melting
KRAS	Kristen Rat Sarcoma
LV	Leucovorin
MAPKs	Mitogen-activated protein kinases
mCRC	metastatic colorectal cancer
OS	Overall survival
PCR	Polymerase chain reaction

PFS	Progression free-survival
RR	Response rate
SNP	Single nucleotide polymorphism
TS	Thymidylate synthase
TTP	Time to progression
US	United States
VEGFR	Vascular endothelial growth factor receptor
VEGF-A	Vascular endothelial growth factor-A
5-FU	5-fluorouracil

Summary

Colorectal cancer (CRC) is the second most common cancer in developed countries, only surpassed by prostate cancer in men and breast cancer in women, and accounts for about 1 million new cases and 530 000 deaths every year. Surgery remains the only curative treatment for patients with this disease, but chemotherapy plays an important role to prolong survival of patients with CRC. With the appearance of new biologic agents that target specific signaling pathways involved in colorectal carcinogenesis, the effectiveness of chemotherapy has greatly increased. Cetuximab and panitumumab are two monoclonal antibodies already approved by the FDA for use in metastatic colorectal cancer (mCRC), which bind selectively to the epidermal growth factor receptor (EGFR) and block ligand induced receptor signaling. Recent clinical trials found an association between *KRAS* mutation *status* and resistance to anti-EGFR therapy, which led to the recommendation of performing *KRAS* mutation analysis prior to cetuximab or panitumumab treatment.

This study was designed to evaluate the efficacy of high resolution melting (HRM) for *KRAS* mutation detection in a clinical setting in comparison to standard DNA sequencing and the DxS Kit, which has been approved for diagnostic use. To accomplish this, a large series of Portuguese patients with mCRC were analyzed and the results of the different methodologies were compared after consensus results were determined with the aid of SNaPshot analysis.

Our results show that DNA sequencing is the least sensitive method for *KRAS* mutation detection (85.71%) and that the sensitivities of HRM (96.90%) and of the DxS Kit (96.15%) do not differ significantly. Furthermore, we show that HRM is the most cost-effective methodology to use in a clinical setting among the ones we tested.

Resumo

O cancro colorectal é o segundo cancro com maior incidência em países desenvolvidos, sendo apenas ultrapassado pelo cancro da próstata no sexo masculino e pelo cancro da mama no sexo feminino, sendo responsável por um milhão de novos casos e 530 000 mortes todos os anos. A cirurgia permanece o único tratamento curativo nos doentes com esta doença, mas a quimioterapia tem um papel importante no aumento da sobrevivência global. Com o desenvolvimento de novos agentes biológicos direccionados a vias de sinalização envolvidas na carcinogénese colorectal, a eficiência da quimioterapia aumentou. O cetuximab e o panitumumab, anticorpos monoclonais já aprovados pela FDA para uso no tratamento de doentes com cancro colorectal metastático, ligam-se selectivamente ao *epidermal growth factor receptor* (EGFR), bloqueando assim esta via de sinalização. Ensaios clínicos recentes encontraram uma associação entre a presença de mutações no gene *KRAS* e resistência a terapia direccionada ao EGFR o que levou a uma recomendação para efectuar a análise de mutações no gene *KRAS* antes do tratamento com cetuximab ou panitumumab.

Este estudo teve como objectivo avaliar a eficácia do uso de *high resolution melting* (HRM) na detecção de mutações no gene *KRAS* em comparação com a sequenciação do DNA *standard* e com o Kit DxS, que foi aprovado para uso diagnóstico. Assim, uma série de doentes portugueses com cancro colorectal metastático foram analisados e os resultados das diferentes metodologias comparados, após um consenso de resultados ter sido obtido com a ajuda da análise por SNaPshot.

Os nossos resultados demonstram que a sequenciação do DNA é a técnica menos sensível para detecção de mutações no gene *KRAS* (sensibilidade de 85,71%), enquanto que o HRM (96,90%) e o Kit DxS (96,15%) apresentam sensibilidades muito semelhantes. Adicionalmente, demonstrámos que o HRM é a metodologia com melhor custo-benefício entre as que foram testadas.

I. Introduction

1.1 Cancer Epidemiology

Cancer is currently the second leading cause of death worldwide with an estimate of 7 million deaths, 12 million new cases and 25 million people alive with cancer in 2008 according to the World Health Organization (WHO). In Europe, in 2008, 3 422 000 new cases of cancer were diagnosed and 1 847 000 people died with the disease (Boyle, 2008). The probability, during life time, of developing invasive cancer is about 44% in men and 39% in women (Jemal *et al.*, 2009). In North Portugal, the cancer registry shows that in 2005 there were 12 950 people (7 219 men and 5 731 women) diagnosed with invasive cancer, corresponding to an overall incidence rate of 394.8/100 000 (RORENO, 2005).

1.2 Carcinogenesis

During development and cellular proliferation there is an intrinsic control system that regulates the balance between cell division and death in response to growth signals and apoptosis. The loss of this regulation is the basis of carcinogenesis. Hence, cancer can be defined as a class of diseases characterized by uncontrolled cell proliferation and also the ability of these cells to spread and metastasize to different tissues. During this process, mutations occur mainly in two groups of genes: proto-oncogenes and tumor suppressor genes. Proto-oncogenes are involved in the regulation of cell proliferation and differentiation and, when mutated, give rise to oncogenes that promote cellular proliferation or impair apoptosis. Tumor suppressor genes are associated with cell growth repression and mutations in these genes cause uncontrolled cell proliferation.

A normal cell needs to acquire six characteristics, which are called hallmarks of cancer, in order to progress to a cancer cell (Hanahan and Weinberg, 2000). These six critical features are: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Recently, Collota *et al* (2009) proposed inflammation as a new hallmark, due to the alterations that provokes in the cellular microenvironment (figure 1). It is thought that all these features are the result of an accumulation of DNA damage. This damage can be caused by many different factors, including environmental factors such as diet or alcohol, or by chemical or physical agents. Being cancer the result of accumulation of DNA damage it is not unexpected that the probability of developing cancer increases with age. Hence, it can be said that the appearance of this disease is due to an interaction between environmental and genetic factors.

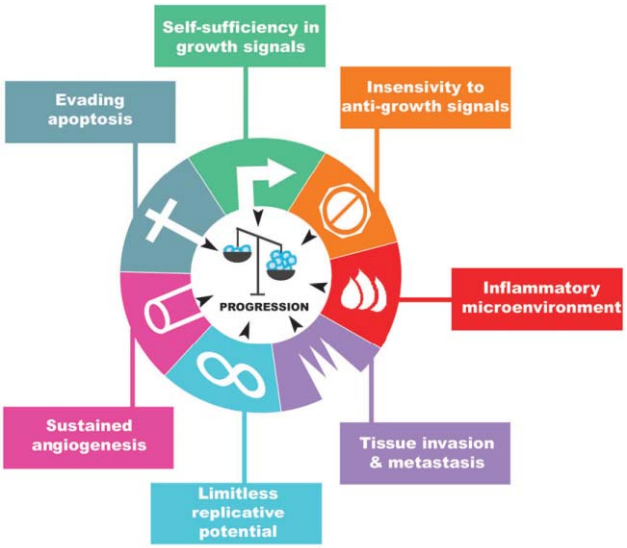

Figure 1 – The seven hallmarks of cancer (Colotta *et al.*, 2009).

1.3 Colorectal cancer

Colorectal cancer (CRC) is the second most common cancer in developed countries (figure 2), only surpassed by prostate cancer in men and breast cancer in women, and accounted for about 1 million new cases in 2002 (9.4% of world total) and 530 000 deaths (7.9% of world total) (Parkin *et al.*, 2005). In Portugal, incidence rates of CRC in 2006 were 58.9/100 000 in men and 30.9/100 000 in women and mortality rates were 30.2/100 000 in men and 17.5/100 000 in women (Ferlay *et al.*, 2007).

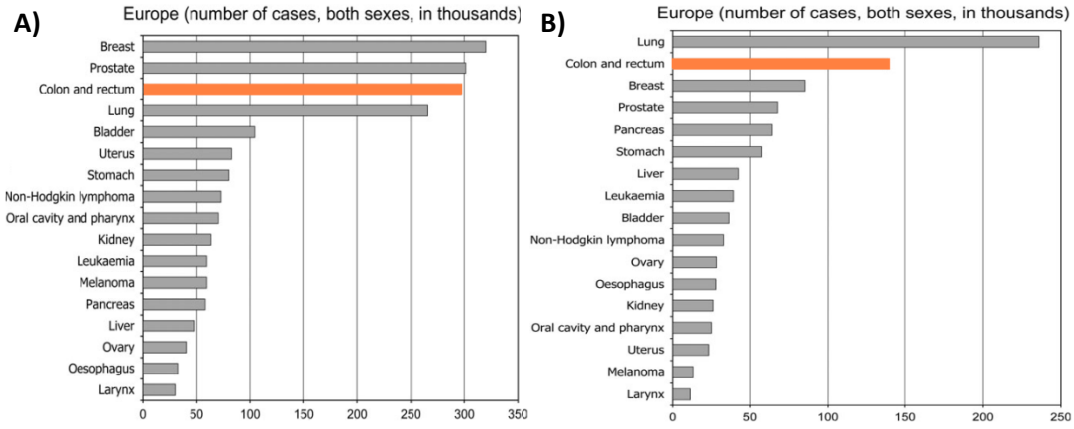

Figure 2 – Incidence **(A)** and mortality **(B)** rates of different cancer types, in both sexes, in Europe, in 2006 (adapted from Ferlay et al., 2007).

Several studies suggest that migrant populations tend to acquire the relative risk of developing CRC of the regions where they migrate to (Haenszel, 1961), which suggests that environmental factors play an important role in the etiology of this disease, being diet one of the most studied (Potter, 1996; Bleiberg, 2002). It is known that a high consumption of lipids and animal proteins is associated with an increased risk of developing CRC (Potter, 1996). In contrast, physical activity appears to decrease the risk of CRC development, probably due to its effect on the intestinal transit, in the metabolism of bile acids and in the immune system (Slattery, 1990). Other environmental factors like obesity, alcohol consumption, tobacco, diabetes and sedentary lifestyle are associated with an increased risk to develop CRC (Le Marchand *et al.*, 1997). The risk of developing CRC is also increased in individuals with chronic inflammatory disease of the colon, such as ulcerative colitis and Crohn's disease (Trichopoulos, 1997; Skibber, 2000).

Individuals with a family history of CRC have an increased risk of developing this disease, especially if the family members were diagnosed before age 45. It is estimated that 5-10% of CRC cases are hereditary and there are two main hereditary syndromes: Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC) (Lynch *et al.*, 1997; Ilyas *et al.*, 1999).

From a clinical point of view, individuals with FAP are characterized by the development of a large number of polyps (>100), showing a cumulative risk of developing CRC by the age of 40 of about 100% (Bishop and Hall, 1994; Midgley and Kerr, 1999). The HNPCC syndrome is associated with early onset of CRC, with carriers of this syndrome presenting an 80% risk at 60 years of developing the disease. These patients also have an increased risk of developing other cancers, including endometrial, stomach and ovary carcinomas (Lynch *et al.*, 1997; Ilyas *et al.*, 1999).

1.4 Colorectal cancer treatment

Surgery remains the only curative treatment for patients with colorectal cancer (Boland *et al.*, 2000). However, 50% of the patients with CRC develop metastases, and most patients with metastatic colorectal cancer (mCRC) eventually die of their disease. Hence, chemotherapy plays an important role in the treatment of patients with metastatic disease and in neoadjuvant therapy to reduce the tumor burden for potentially resectable disease (Chu, 2008).

In general, patients with mCRC who are not treated have a median survival of 5-6 months. With the development of the first chemotherapeutic agent, 5-fluorouracil (5-FU), the median survival was extended to 11-12 months (Chu, 2008). More recently, clinical trials with combination therapy and biologic agents showed a median survival of 20-24 months in patients with mCRC, something that demonstrates the impact of chemotherapy on patient survival (Chu, 2008). There are currently three cytotoxic agents used alone or in combination therapy (5-FU, oxaliplatin and irinotecan) and another three biologic agents (bevacizumab, cetuximab and panitumumab) used in the treatment of mCRC.

5-FU was first synthesized in the late 1950s and was for a long time the only available drug to treat mCRC. It is inactive in its patent form and converted within the cell to the cytotoxic metabolite fluorodeoxyuridine monophosphate (FdUMP) (Huang and Ratain, 2009). FdUMP blocks the enzyme thimidylate synthase (TS) through the formation of a ternary complex, preventing the synthesis of thymine nucleotides and DNA replication. 5-FU can also be falsely incorporated into RNA and DNA, which leads to inhibition of mRNA translation, protein synthesis and DNA synthesis (Huang and Ratain, 2009). Different methods of administering 5-FU and different agents as biochemical modulators of 5-FU were tested to improve response rates (RR), but currently the most commonly combination used is infusional administration of 5-FU together with the vitamin Leucovorin (LV), which increases the intracellular pool of reduced folates and stabilizes the interaction between FdUMP and TS. A meta-analysis of individual patient data reported a 23% RR on patients administered with infusional 5-FU and LV (Project, 1992; Segal and Saltz, 2009) and this was the standard therapy used in patients with mCRC for more than 20 years. Capecitabine is an oral fluoropyrimidine prodrug that is converted to 5-FU by thymidine phosphorylase at the site of the tumor. The efficacy of this agent is similar to that observed with 5-FU plus LV, as was demonstrated in a meta-analysis of individual patient data from two large randomized phase III trials (Van Cutsem *et al.*, 2004; Chau and Cunningham, 2009). Therefore, currently both capecitabine and 5-FU serve as the basis of combination therapy that integrates other cytotoxic and biologic agents in the treatment of mCRC.

Irinotecan is a camptothecin analog that inhibits DNA topoisomerase I and induces single-strand DNA breaks and replication arrest through the action of its active metabolite, SN-38 (Huang and Ratain, 2009). Irinotecan is currently used either as a single agent, in combination with 5-FU/LV (FOLFIRI) or with cetuximab. A phase III European clinical trial compared FOLFIRI to infusional 5-FU plus LV alone and found out that FOLFIRI was

superior in response rate (35% *vs* 22%, $p<0.005$) and overall survival (OS) (17.4 *vs* 14.1 months, $p=0.031$) (Douillard *et al.*, 2000; Wolpin and Mayer, 2008).

Oxaliplatin is a platinum compound that forms cross-linking DNA adducts, thus blocking DNA replication and inducing apoptotic cell death (Huang and Ratain, 2009). It is usually administered in combination with 5-FU/LV (FOLFOX) or with Capecitabine (CAPOX) since this agent has no effect when used as a single agent. Gramont *et al* (2000) reported the first comparison between FOLFOX and 5-FU plus LV, which showed a statistical significant advantage of FOLFOX (progression free-survival (PFS) 9 *vs* 6.2 months and RR 50.7% *vs* 22.3%). More recently, Tournigand *et al* (2004), conducted a phase III trial to address the question of which combination treatment would benefit more patients with mCRC. In order to do this, patients were divided in two arms: one received FOLFIRI and the other one FOLFOX. At the time of progression the treatment was reversed and patients who received FOLFIRI as first-line therapy now received FOLFOX and *vice-versa*. The RRs for both arms were almost identical (56% *vs* 54%) as well as the overall median survival (20.4 *vs* 21.5 months). This study is very important because it shows that both treatment combinations have equivalent efficacy and also that the order which they are given yields no difference in overall survival.

A triplet combination (5-FU, Irinotecan and Oxaliplatin) has also been tested but the results show similar RRs and time to progression (TTP) with an increased toxicity. Nevertheless, it is known that exposure of patients to all three cytotoxic agents during their treatment course increases OS (Grothey *et al.*, 2004; Chu, 2008; Segal and Saltz, 2009).

More recently, “targeted” agents have been developed for use in mCRC treatment, in particular, agents targeting two key cellular processes: the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGFR). Bevacizumab is a humanized monoclonal antibody that inhibits blood-vessel formation by binding to vascular endothelial growth factor-A (VEGF-A), a key regulator of normal and tumor-associated angiogenesis (Carmeliet, 2005). When tumor size is still low, tumor cells are able to obtain the required nutrients and oxygen from surrounding fluids via diffusion, but as the size of the tumor increases new blood vessels must be formed and VEGF is a key component in the formation of these new blood vessels (Carmeliet, 2005). The first study where a potential role of bevacizumab in mCRC was tested was a phase II trial that administered different doses of bevacizumab plus 5-FU and LV. The results showed an improved RR and TTP in the patients that were given 5 mg/kg of bevacizumab. No significant toxicity was observed (Kabbinavar *et al.*, 2003; Segal and Saltz, 2009). In the phase III study (Hurwitz *et al.*, 2004; Segal and Saltz, 2009), patients were treated with

irinotecan plus 5-FU and LV and bevacizumab or placebo. Bevacizumab was found to increase the RR (34.8% *vs* 44.8%), PFS (6.2 *vs* 10.2 months) and OS (15.6 *vs* 20.3 months), which lead to the approval of this drug as first-line treatment in mCRC by the United States (US) Food and Drug Administration (FDA) in 2004.

Cetuximab is a chimeric (human/mouse) immunoglobulin IgG1 monoclonal antibody that binds selectively to the EGFR and blocks ligand induced receptor signaling (Mendelsohn and Baselga, 2003). The first experiments done with cetuximab suggested that it had greater activity combined with cyotoxic therapy compared to being used as a single agent. Two clinical trials were very important to establish the efficacy of cetuximab in the treatment of mCRC, the BOND (Cunningham *et al.*, 2004) and the CRYSTAL (Van Cutsem *et al.*, 2007) trials. In the BOND trial, 329 patients with irinotecan-refractory mCRC were randomized in two arms: one receiving cetuximab and irinotecan and other cetuximab alone. The group that received the combination therapy had an overall RR of 23% against 11% in the patients treated with cetuximab alone. Time to tumor progression also had significative differences (4.0 *vs* 1.6 months), but no such effect was observed in overall survival. In the CRYSTAL trial, patients received FOLFIRI or FOLFIRI plus cetuximab in first-line therapy. The addition of cetuximab resulted in a statistically significant, albeit extremely modest, improvement in PFS, the primary endpoint of the trial (8.9 *vs* 8.0 months, p=0.048), as well as an improvement in RR (46.9% *vs* 38.7%, p=0.005).

Panitumumab is a fully human IgG2 monoclonal antibody with similar activity to cetuximab. A phase III clinical trial compared panitumumab against best supportive care (BSC) and demonstrated the efficacy of this biologic agent with a significantly longer PFS (8 *vs* 7.3 weeks), albeit median OS was not improved in panitumumab-treated patients (Van Cutsem *et al.*, 2007; Chu, 2008), which lead to the approval of this drug by the FDA for use as a single agent after relapse. It is currently under investigation the role of panitumumab with combination therapy (FOLFOX and FOLFIRI), but it is thought that it will have comparable efficacy to cetuximab due to the very similar in structure and function. Thus, there is no basis to use one after the failure of a previous treatment with the other.

Current practice in the US and Europe is to use cytotoxic regimens such as FOLFIRI and FOLFOX in the front-line setting in combination with bevacizumab, albeit recent data suggest that cetuximab can also have an important effect in first-line therapy. During the course of the disease, if patients relapse, it is important to integrate the different available agents to obtain prolonged survival and the choice of the best treatment usually depends on three factors: the type of therapy the patient received and the response, the patient's

comorbidities and the goal of the therapy in terms of curative *versus* palliative intentions (figure 3).

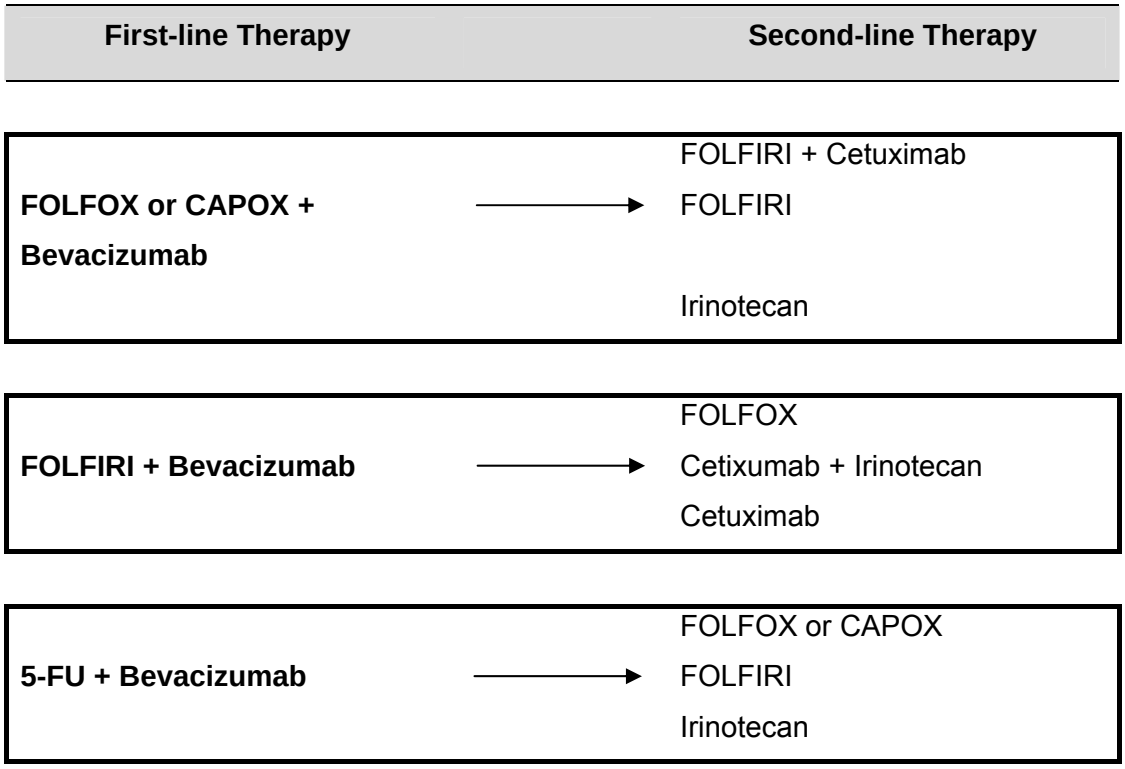

Figure 3 – Algorithm for metastatic colorectal cancer treatment (adapted from Chu, 2008)

1.5 Biology of colorectal cancer

The first multistep genetic model of colorectal carcinogenesis was proposed in 1990 by Fearon and Vogelstein, based on the observation of specific genetic changes in benign and malignant lesions (Fearon and Vogelstein, 1990). Some of the key features of this model are mutational activation of a large number of oncogenes and mutation inactivation of tumor suppressor genes, the necessity of mutations in at least four or five genes for tumor formation and the fact that the order in which these mutations occur is less relevant than the total accumulation of genetic mutations (figure 4).

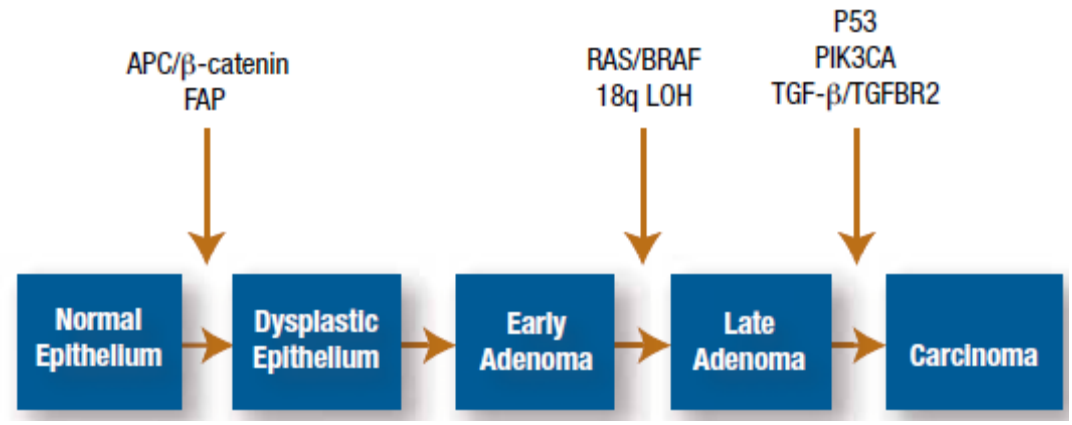

Figure 4 – Genetic model of colorectal carcinogenesis (Chu, 2008)

These mutations that are necessary for the initiation and tumor progression in CRC occur among a variety of genes, such as *APC*, *KRAS* or *TP53*. Mutations in *APC* are an early step on CRC carcinogenesis and mutation frequency in sporadic adenomas varies from 30-70%, whereas in colorectal carcinomas varies from 60-80% (Ilyas *et al.*, 1999; Worthley *et al.*, 2007). The function of the protein encoded by *APC* gene is to inhibit β-Catenin and regulate the Wnt signaling pathway. Another gene frequently mutated in CRC is *TP53* and mutations in this gene are associated with the transition between late adenoma to carcinoma. The frequency of mutation in this gene in CRC is 35-75% (Smith *et al.*, 2002). The protein encoded by this gene (p53) is an important transcription factor that mediates cell cycle arrest in order to facilitate DNA repair during replication, or induces apoptosis if it is not possible to repair the DNA (Leslie *et al.*, 2002; Smith *et al.*, 2002; Worthley *et al.*, 2007). Another important event in CRC carcinogenesis is the occurrence of 18q deletion in 60 to 70% of CRC cases (Liu, 2003). In this chromosomal region several important genes are located, such as *SMAD2* and *SMAD4*. These genes are involved in the Transforming Growth Factor β (TGFβ) signaling pathway, an important pathway in the control of cell proliferation, differentiation, migration and apoptosis. Frameshift mutations in the *TGFBR2* are present in up to 80% of CRC with microsatellite instability (Xu and Pasche, 2007).

The Kristen Rat Sarcoma (*KRAS*) proto-oncogene encodes a signal transduction protein, which in its active state forms a complex with a guanosine triphosphate (GTP) group. This complex is inactivated by hydrolysis of GTP to guanosine diphosphate (GDP). The frequency of mutations in the *KRAS* proto-oncogene in sporadic CRC is 30 to 50% (Smith *et al.*, 2002; Calistri *et al.*, 2005), but can be as high as 90% in pancreatic cancer. The most common mutations found in CRC are in exon 2 and to a lesser extent in exon 3 (Calistri *et al.*, 2005). If *KRAS* is mutated, the resulting complex is less sensitive to

hydrolysis, remaining in a constitutively active state, leading to cell proliferation by a variety of signaling pathways, including the Mitogen-activated protein kinases (MAPKs) pathway (Arends, 2000; Takayama *et al.*, 2006). A high frequency of mutations in this gene in benign lesions suggests that, although providing a selective growth advantage to cells, it is not sufficient by itself to trigger carcinogenesis. The accumulation of mutations in this gene and others, including the *APC* gene, will presumably give a selective advantage to the mutated cells, resulting in their clonal proliferation (Worthley *et al.*, 2007).

When KRAS is activated it induces the MAPK signal transduction cascade, transferring signals from the cell membrane to the nucleus. The proteins encoded by the RAS gene activate RAF family proteins, including V-RAF 3611 Murine Sarcoma Viral Oncogene Homolog (ARAF), V-RAF Murine Sarcoma Viral Oncogene Homolog B1 (BRAF) and V-RAF-1 Murine Leukemia Viral Oncogene Homolog 1 (RAF-1), which are known as MAP kinase kinase kinases (MAPKKKs) because of its function to phosphorylate and activate the MAP kinase kinases (MAPKKs) MEK-1 and MEK-2. The MAPKKs subsequently phosphorylate the MAPKs ERK-1 and ERK-2, which in turn phosphorylate nuclear substrates such as the transcription factors V-JUN Avian sarcoma virus 17 oncogene homolog (JUN) and Member of ETS Oncogene Family (ELK1) (figure 5). The activation of these transcription factors leads to the expression of proteins that control the cell cycle (Kerkhoff and Rapp, 1998; Leslie *et al.*, 2002; Chong *et al.*, 2003).

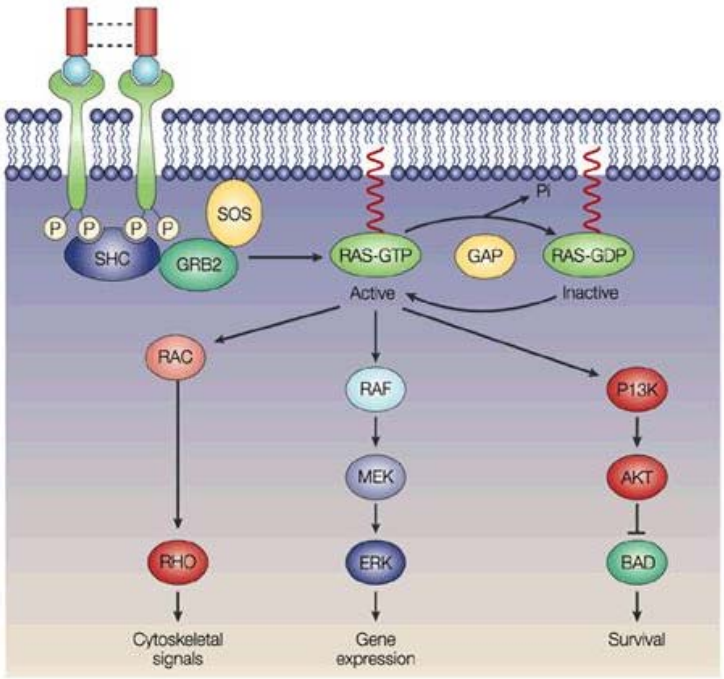

Figure 5 – Ras signalling pathway (Stirewalt and Radich, 2003).

As stated previously, the addition of biologic agents that target specific signaling pathways involved in colon carcinogenesis improved significantly the RR and OS. However, 40-50% of mCRC patients either do not experience clinical benefit with these therapeutic approaches or suffer from severe toxicity. Hence, the search for molecular markers that could predict the response to these drugs and improve clinical benefit was initiated.

The *KRAS* gene was one of the first studied due to its involvement in CRC carcinogenesis. A retrospective study analyzed *KRAS*, *BRAF* and *TP53* mutation status and their association with therapeutic response and survival in patients treated with bevacizumab but no associations were found (Jain *et al.*, 2006). The first retrospective studies that evaluated *KRAS* mutation status in patients treated with cetuximab or panitumumab revealed a significant association of favorable response in patients with *KRAS* wild-type, with RRs of 17-48%, but no response in patients with a mutation in *KRAS* codon 12 or 13 (Benvenuti *et al.*, 2007; Di Fiore *et al.*, 2007; Khambata-Ford *et al.*, 2007; Amado *et al.*, 2008; De Roock *et al.*, 2008; Lievre *et al.*, 2008).

The CRYSTAL and the OPUS clinical trials were the first to prospectively analyze *KRAS* mutational status and clinical response to cetuximab (Van Cutsem *et al.*, 2007; Bokemeyer *et al.*, 2009). CRYSTAL trial patients were treated with FOLFIRI alone or FOLFIRI+Cetuximab, whereas OPUS trial patients were treated with FOLFOX alone or FOLFOX+Cetuximab. A significative association between response to cetuximab treatment and *KRAS* wild-type status was observed in both studies, with no benefit observed in patients that had a *KRAS* mutation. This association was observed both in RR and progression-free survival. With this evidence, the American Society of Clinical Oncology released a statement in early 2009, recommending that all patients who are candidates for anti-EGFR antibody treatment should be tested for *KRAS* mutation *status* prior to the start of the treatment (Allegra *et al.*, 2009).

BRAF is a gene downstream of *KRAS* in the MAPK pathway that is also commonly mutated in CRC, although to a less extent. Mutations in these two genes are mutually exclusive (Rajagopalan *et al.*, 2002) and therefore BRAF could be a locus for a second hit affecting the same pathway. It is now known that mutations in BRAF also confer resistance to anti-EGFR therapy. A clinical trial with 11 patients positive for the mutation V600E in *BRAF* demonstrated that they do not respond to treatment with cetuximab or panitumumab (Di Nicolantonio *et al.*, 2008). Another clinical trial analyzing 13 patients with *BRAF* V600E mutation found a significative association between mutations in this gene

and resistance to cetuximab treatment (Loupakis *et al.*, 2009). The 13 patients positive for mutations did not respond to treatment *vs* 24 out of 74 wild-type patients (p=0.016). As a result, it is likely that not only *KRAS* but also *BRAF* mutation analysis will be mandatory in patients with mCRC prior to anti-EGFR therapy.

Despite some evidence of other alterations possibly being predictive markers of response to the different cytotoxic agents currently used in mCRC, analysis of *KRAS* mutation *status* has been the only genetic testing widely used prior to anti-EGFR therapy, since 2008.

1.6 KRAS mutation detection

Detection of mutations in the *KRAS* gene is now standard practice prior to anti-EGF therapy. Hence, it is important to have a reliable, fast and cheap clinical test for the detection of these mutations. There are now a number of different methodologies that can be used for detection of DNA mutations, but the "gold standard" for this type of analysis for a long time has been DNA sequencing. However, when dealing with somatic mutations, the mutant alleles need to be present in at least 20% of the tumor sample to allow its detection with this methodology (Do *et al.*, 2008). Recently, new methodologies have emerged that may provide a faster and more reliable analysis. It should be noted that for detection of *KRAS* mutations, most often archival paraffin-embedded tissue samples are used, hence the methodology used for DNA extraction is also very important. Since the sensitivity and specificity of each methodology is influenced by the proportion of cancer cells in the sample, most of the times macrodissection is used to enrich the sample of tumor cells and eliminate normal cells (Whitehall *et al.*, 2009).

One of the new methodologies currently being used for detection of DNA mutations is high resolution melting (HRM). It was first introduced in 1997, but at that time its sensitivity was not enough to detect subtle sequence variations. With the introduction of saturating dyes like LCGreen or EvaGreen and the development of specialized equipment, the sensitivity and specificity for detection of DNA alterations greatly increased. The sensitivity to detect heterozygous germline mutations is close to 100% (Takano *et al.*, 2008) and for detection of somatic mutations in tumors it has been shown to be able to detect mutant alleles present at levels as low as 5% (Krypuy *et al.*, 2006). This methodology is based on the melting pattern of individual amplicons as they are submitted to an increase in temperature after polymerase chain reaction (PCR)

amplification. Application of this methodology varies from mutation screening (Krypuy *et al.*, 2007; De Leeneer *et al.*, 2008; Rouleau *et al.*, 2009; van der Stoep *et al.*, 2009), single nucleotide polymorphism (SNP) genotyping (Reed and Wittwer, 2004; Erali *et al.*, 2008) and methylation analysis (Worm *et al.*, 2001; Maat *et al.*, 2007). One of the main disadvantages of HRM is the fact that it does not give sequence information, requiring DNA sequencing or other methodology for confirmation and genotyping of unknown mutations.

The DxS *KRAS* mutation detection kit is a real-time PCR-based assay based on ARMS® and Scorpions® technology. Using ARMS technology, it is possible to perform allele or mutation-specific amplification even in samples where the majority of the sequences do not carry the mutation. Scorpions is the technology used for detection of amplification, which is by linking a PCR primer to a probe. The fluorophore in this probe interacts with a quencher, also incorporated into the probe, which reduces fluorescence. During a PCR reaction, when the probe binds to the amplicon, the fluorophore and quencher become separated. This leads to an increase in fluorescence from the reaction tube that is detected by a real-time PCR system. The DxS Kit, which has been approved for KRAS mutation detection in the USA and Europe, only detects and identifies the seven most common mutations in codons 12 and 13. It has a high sensitivity for detection of those seven mutations, being possible to detect a mutant allele present in at least 1% of all cells in a tumor sample (Monzon *et al.*, 2009), but it is a very expensive methodology.

The SNaPshot methodology was first developed for genotyping of SNPs, but it is now also widely used for detection of common mutations in a variety of different genes (van Oers *et al.*, 2005; Filippini *et al.*, 2007). This method offers a specific, sensitive, relatively inexpensive and rapid alternative for mutation screening. It has been reported as able to detect a mutation present in 5% or less of the total cells in tissue samples (Hurst *et al.*, 2009).

II. Objectives

The main objectives of this study were:

- Determination of *KRAS* exon 2 mutation frequency in a large series of Portuguese patients with metastatic colorectal cancer;

- Evaluation of three different methodologies (DNA sequencing, DxS Kit, HRM) for *KRAS* mutation detection in a clinical setting using SNaPshot as a fourth method to define consensus findings when necessary;

- Cost-effectiveness analysis of the different methodologies.

III. Materials and Methods

3.1 Samples

A total of 300 consecutive patients (97 women, 203 men) with metastatic colorectal cancer (mCRC) referred to the Genetics Department of IPO Porto for *KRAS* codon 12 and 13 mutation analysis were studied by either DNA sequencing or the DxS Kit and by HRM. SNaPshot was used as a fourth method to define consensus findings when necessary. Written informed consent was obtained from all patients prior to the testing.

3.2 DNA extraction from formalin-fixed paraffin-embedded tissue

Hematoxylin and eosin (H&E) stained slides were carefully reviewed by a pathologist, who delimited areas with >50% tumor cells. Tissue sections of 10 µm thickness were obtained from formalin-fixed paraffin-embedded (FFPE) tissue and placed on 0.1% Poly-L-lysine coated slides [Sigma Chemical Co.®] and dried at 37°C overnight. Overlapping the 10 µm thick slides with the H&E stained slides, tumor areas were delimited on the first. Slides were then soaked on xylene [SIGMA] for 10 minutes and twice on a 100% ethanol [Merck] solution for 5 minutes to remove the paraffin. Tumor areas were carefully scraped using a sterile scalpel blade and then transferred to a microcentrifuge tube. Lysis buffer (1 mL total volume) was prepared with 50 µL of Tris-HCl [SIGMA] 1M (pH 8.5), 10 µL of EDTA [E-5134], 5 µL of Tween 20 [GIBCO BRL] and 935 µL of distilled water [Braun]. Two hundred microliters of Lysis buffer and 30 µl of proteinase K [GIBCO BRL] (10 mg/mL) were added, followed by incubation at 55°C overnight.

On the following day, 250 µL of phenol/chloroform [Sigma]/[Merck] was added to the microcentrifuge tube, which was then vortexed and centrifuged at 13000 rpm [Eppendorf] for 5 min. Supernatant was transferred to another tube and the double supernatant volume of 100% ethanol and 1/3 the supernatant volume of ammonium acetate [SIGMA] 7.5M was added. The tube was left overnight at -20°C and centrifuged in the following day at 14000 rpm for 12 min. Supernatant was discarded and the pellet was washed in 70% ethanol twice followed by a 12 min centrifugation at 13000 rpm. Pellets were then left to dry at 37°C and eluted in 80 µL of distilled water (Pearson and Stirling, 2003). DNA quality was evaluated using NanoDrop ND-1000® [NanoDrop Technologies].

3.3 DNA Sequencing

Mutation screening in *KRAS* exon 2 in 185 patients with mCRC was done according to Sanger DNA sequencing method.

First, PCR reaction was done using 1x Taq reaction buffer [Perkin-Elmer] (10 mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$ [Perkin-Elmer], 250 µM of dNTPs [Perkin-Elmer], 500 nm of each primer, 0.2 U of Taq DNA polymerase [Perkin-Elmer], 30 to 50 ng of DNA and distilled water in a total reaction volume of 20 µL. The primers used were GACTGAATATAAACTTGTGG (forward) and CTATTGTTGGATCATATTCG (reverse) as described by Davies et al (2002).

PCR reaction was performed in a termocycler [Perkin-Elmer, Gene Amp PCR Systern 9700] according to the following conditions: an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing step at 55°C for 1 min and a 2 min extension step at 72°C. A final extension step was done at 72°C for 10 min. Amplified products were then analyzed by electrophoresis in a 2% (w/v) agarose gel [GIBCO BRL] stained with ethidium bromide [SIGMA] 5 µg/µL.

PCR product was purified with GFX PCR DNA and Gel Band Purification Kit [GE Healthcare] to remove salts, enzymes, nucleotides and non-incorporated primers. Samples were purified according to manufacturer's protocol and eluted in distilled water. Purified PCR products were quantified by electrophoresis using a 2% (w/v) agarose gel stained with ethidium bromide 5 µg/µL.

On the sequencing reaction 30 to 90 ng of purified DNA were used together with 1µL of Terminator Ready Reaction Mix [Applied Biosystems] (dNTPs, ddNTPs-fluorocromes, $MgCl_2$, Tris-HCl buffer), 1.9 µL of sequencing buffer [Applied Biosystems], 350 nm of primer and distilled water to reach a total volume of 10 µL. The PCR reaction was carried out in a termocycler and consisted of an initial denaturation step at 96°C for 5 min, followed by 35 cycles of denaturation at 96°C for 10 seconds, annealing at 52°C for 5 seconds and extension at 60°C for 4 min.

The sequencing product was purified in order to remove excess of dNTPs, labeled ddNTPs and non-incorporated primers. 2 µL of sodium acetate [Merck] and 50 µL of ethanol 96% (v/v) were added to the sequencing products and the tube was vortexed and placed on ice for 30 min. Next, the sample was centrifuged at 14000 rpm for 30 min at 4°C. Supernatant was discarded and 250 µL of ethanol 70% (v/v) were added to wash the

DNA. After centrifugation at 14000 rpm for 20 min, the supernatant was discarded and the sample was dried at room temperature.

The precipitate was eluted in deionized formamide [Applied Biosystems] and, after denaturation at 95°C for 5 min, the sample was placed in an automatic sequencer ABI PRISM 310 Genetic Analyzer [Applied Biosystems], where sequencing products were subjected to capillary electrophoresis. The different oligonucleotides migrate according to their molecular weight and the device registers their emission spectrum constructing a diagram that after analysis indicates the DNA sequence.

3.4 DxS Kit

Mutation analysis for 115 samples was performed using the DxS: *K-RAS* mutation detection kit [DxS Diagnostic Innovations], following the manufacturer's protocol. This technology combines allele specific PCR (ARMS) with real-time PCR (Scorpions) to detect the seven most common mutations at KRAS codon 12 and 13 (p.Gly12Ala, p.Gly12Asp, p.Gly12Arg, p.Gly12Cys, p.Gly12Ser, p.Gly12Val, p.Gly13Asp). Mutation detection was done using a LightCycler®480 II Real-Time System [Roche Diagnostics] and the LightCycler® Adapt Software v1.1 [Roche Diagnostics].

3.5 High Resolution Melting

PCR and HRM analysis were performed in the LightCycler®480 II Real-Time System [Roche Diagnostics]. The primers used to amplify a 92 bp amplicon of *KRAS* exon 2 were the following: TTATAAGGCCTGCTGAAAATGACTGAA (forward) and TGAATTAGCTGTATCGTCAAGGCACT (reverse), as described by Krypuy et al (2002). PCR reaction included 1x LightScanner® Master Mix [Idaho], 150 nM of each primer, 50 ng/µL of DNA and distilled water in a total reaction volume of 10 µL. Fifteen microliters of mineral oil [Sigma] were added to all wells in the plate to prevent evaporation and cross-contamination. Cycling and melting conditions were the following: one cycle of 95°C for 15 min; 38 cycles of 95°C for 15 seconds, 69°C for 15 seconds and 72°C for 15 seconds; an heteroduplex step of 95°C for 1 min and 40°C for 1 min; and a melt from 75°C to 90°C with 25 acquisitions per second. All samples were run in duplicate and melt curve analysis was done using LightCycler® 480 Software [Roche Diagnostics], version 1.5 with 0.3 sensitivity. Normalization areas were 80°C-81°C pre-melt and 85.5°C-87°C post-melt. Amplification

plots were used to analyze quality of the DNA samples and only those with a Ct<30 and a sigmoid curve were considered. The normalized graph was generated by monitoring the dissociation of the fluorescent dye from the double-stranded DNA as temperature increased. The fluorescent dye used (LCGreen® Plus [Idaho]) is a saturating dye, meaning that only intercalates double-stranded DNA and at a saturating concentration. The difference graph was used to differentiate between wild-type samples and samples containing a mutation in *KRAS* exon 2. It is generated by comparison of all melting curves and conversion of a wild-type melting profile into a horizontal line. Significant deviations from the horizontal line were indicative of presence of a mutation in the amplicon. All samples with a result different from the one obtained through DNA sequencing or the DxS Kit were sequenced directly from the HRM product according to the protocol already described.

3.6 SNaPshot

A SNaPshot assay was developed for mutation detection in samples with a non-consensus result. SNaPshot chemistry is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer. The primer is designed to anneal to the sequence adjacent to the nucleotide of interest. Once the primer anneals, the single-base extension occurs by the addition of the complementary dye-labelled ddNTP (dye terminator) to the annealed primer. Each of the four ddNTPs is fluorescently labelled with a different colour dye. The result is marker fragments for the different SNP alleles that are all the same length, but vary by colour. After electrophoresis and fluorescence detection, the alleles of a single marker appear as different colored peaks in the electropherogram plot.

Using a Multiplex SNaPshot reaction it is possible to detect all mutations at the four nucleotides (c.34, c.35, c.37 and c.38) that can be mutated in codon 12 and 13 of *KRAS* exon 2. PCR amplification was performed as described above for DNA sequencing, with the same conditions and primers. Multiplex SNaPshot reaction [Applied Biosystems] and capillary electrophoresis was done following the manufacturer's protocol. The primers used in the multiplex reaction to detect mutations at the four nucleotides of interest were the following:

c.34: 5-AACTTGTGGTAGTTGGAGCT-3´;

c.35: 5-GACTGAACTTGTGGTAGTTGGAGCTG-3´;

c.37: 5'-GACTGACTGACTTTGTGGTAGTTGGAGCTGGT-3';

c.38: 5-GACTGACTGACTGACTGATGTGGTAGTTGGAGCTGGTG-3'

IV. Results

In order to analyze the three different methodologies (DNA sequencing, the DxS Kit and HRM) for KRAS mutation detection, 300 tumor samples were analyzed by HRM, while 185 of those were analyzed by DNA sequencing and the other 115 with the DxS Kit. Results of DNA sequencing and the DxS Kit were compared with those of HRM and cases with a discrepant result were further analyzed by SNaPshot. A consensus result was considered when two out of three methodologies (HRM, DNA sequencing and SnapShot or HRM, DxS Kit and SnapShot) gave a similar result.

4.1 DNA sequencing

A total number of 185 (62 women, 123 men) tumor DNA samples were analyzed by DNA sequencing. Of these, 117 (63.24%) were considered as wild-type for *KRAS* exon 2 mutations, 66 as positive (35.68%) and in two cases (1.08%) it was not possible to obtain data because DNA quality was poor for this methodology. Results are detailed in table 1 and examples of wild-type samples and samples with mutations in codon 12 and 13 are shown in figure 6.

Table 1 – DNA sequencing results of 185 samples

Result	Nº of cases	%
Wild-type	117	63.24
p.Gly12Asp	21	11.35
p.Gly12Val	20	10.81
p.Gly13Asp	11	5.95
p.Gly12Ala	7	3.78
p.Gly12Cys	3	1.62
p.Gly12Ser	2	1.08
p.Gly12Phe	1	0.54
p.Gly13Cys	1	0.54
ND	2	1.08

ND – No Data

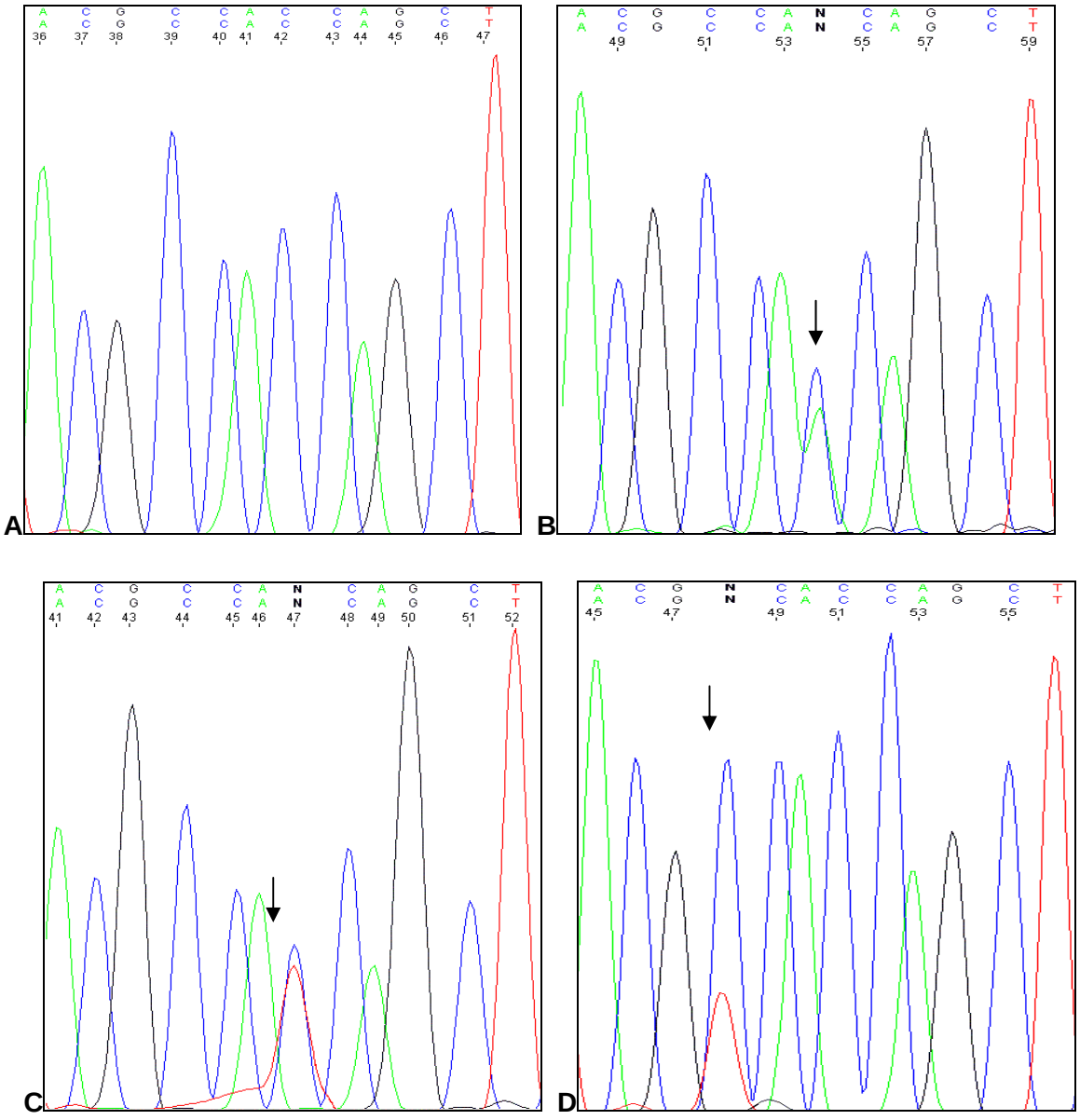

Figure 6 – Electropherograms of a wild-type sample **(A)** and positive samples for the p.Gly12Val **(B)**, p.Gly12Asp **(C)** and p.Gly13Asp **(D)** mutations. All electropherograms presented are the reverse strand.

4.2 DxS Kit

The DxS Kit was used in 115 (35 women, 80 men) DNA samples to detect the seven most common mutations in codons 12 and 13 of *KRAS* exon 2. Of these, 63 (54.78%) were considered as wild-type, 50 as positive (43.48%) and in two cases (1.74%) it was not possible to obtain data because DNA quality was poor for this methodology. Results are

detailed in table 2 and examples of a wild-type sample and a sample with a mutation are shown in figure 7.

Table 2 – DxS Kit results of 115 samples

Result	Nº of cases	%
Wild-type	63	54.78
p.Gly12Val	20	17.39
p.Gly12Asp	11	9.57
p.Gly13Asp	8	6.96
p.Gly12Cys	5	4.35
p.Gly12Ala	2	1.74
p.Gly12Arg	2	1.74
p.Gly12Ser	2	1.74
ND	2	1.74

ND – No Data

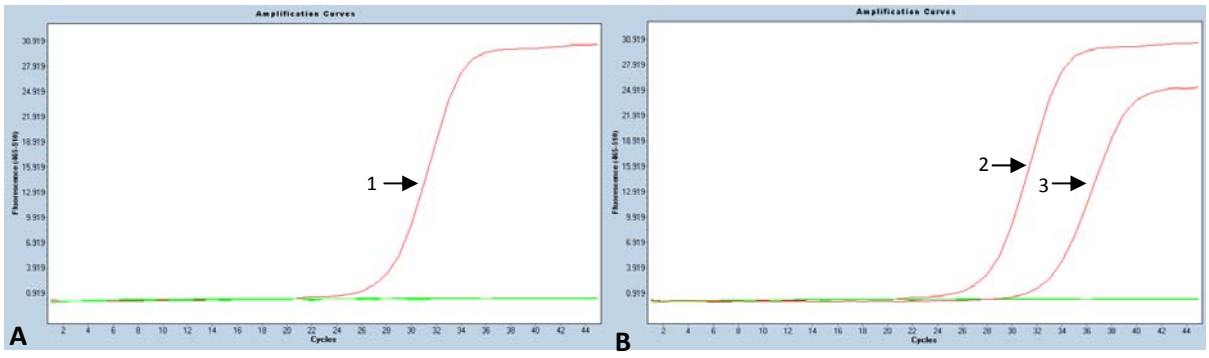

Figure 7 – Amplification plots of a wild-type sample **(A)** and a mutation positive sample **(B)** with DxS Kit. Arrows 1 and 2 show amplification of the DNA quality control and arrow 3 amplification of a p.Gly12Val mutation.

4.3 High Resolution Melting

Of the 300 (97 women, 203 men) DNA samples analyzed by HRM, 173 (57.67%) were considered as wild-type and 127 (42.33%) positive for mutations in *KRAS* exon 2. Results are detailed in table 3 and examples of an amplification plot, a normalized graph and a difference graph of wild-type samples and samples with mutations are shown in figure 8. It was possible to obtain a result in all samples.

Table 3 – HRM results in 300 samples

Result	Nº of cases	%
Wild-type	173	57.67
Positive	127	42.33

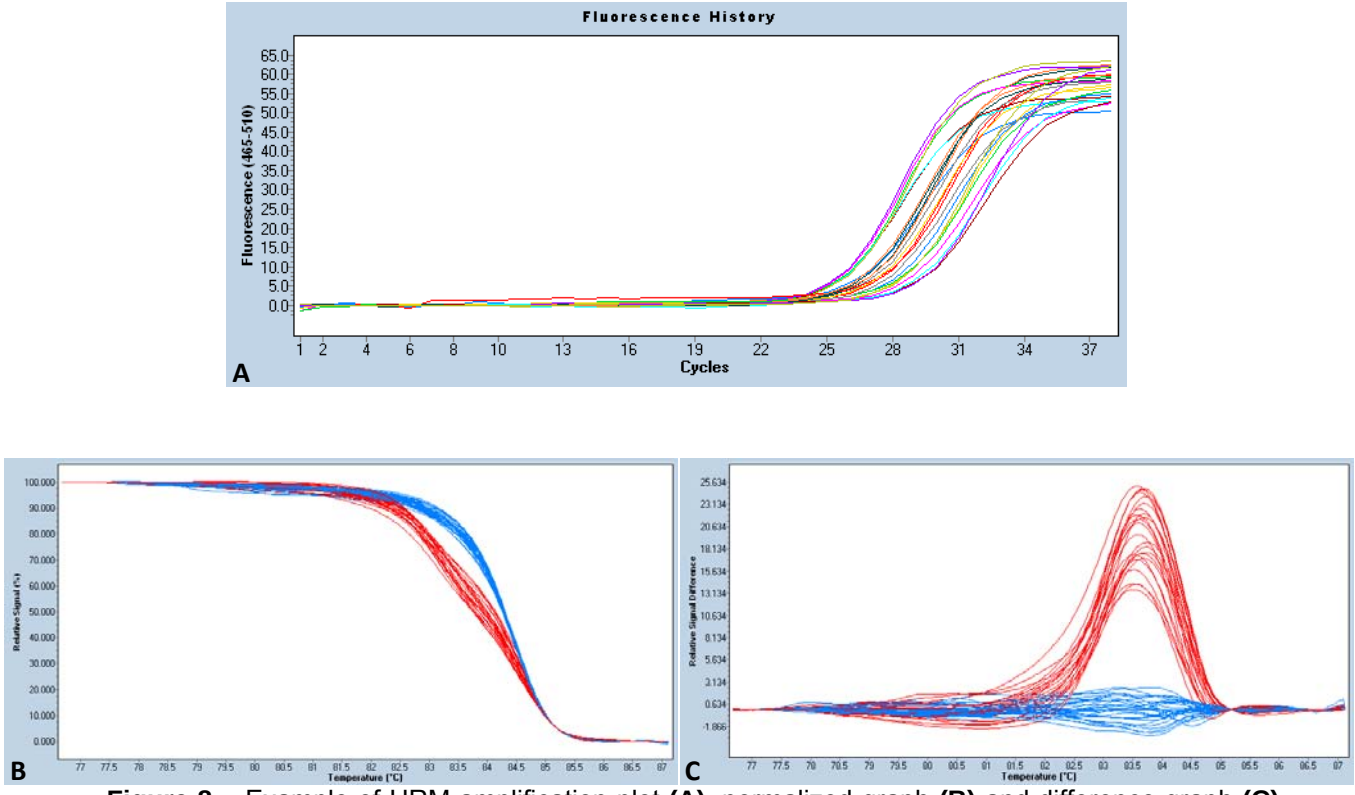

Figure 8 – Example of HRM amplification plot **(A)**, normalized graph **(B)** and difference graph **(C)** containing wild-type **(blue)** and positive **(red)** samples.

4.4 HRM vs DNA sequencing

KRAS mutation status results by HRM and DNA sequencing are shown in table 4. In 12 samples a consensus result was not possible to obtain and all of them were considered wild-type by DNA sequencing and positive for mutations by HRM. The two samples that were not possible to analyze by DNA sequencing were analyzable by HRM, one being considered wild-type and the other as positive.

Table 4 – Comparison between HRM and DNA sequencing results.

Result	HRM	DNA sequencing
Wild-type	105	117
Positive	78	66

In the 12 samples in which a consensus result was not obtained between DNA sequencing and HRM, sequencing of the HRM products was performed. Results of HRM products sequencing indicated that 11 of the samples had a mutation in either codon 12 or 13 of *KRAS* exon 2 and one sample was wild-type (table 5).

Table 5 – Comparison of results between HRM, DNA sequencing and sequencing of HRM products in the 12 non-concordant samples.

Case Nº	DNA sequencing	HRM	HRM sequencing
1	Wild-type	Positive	p.Gly12Asp
2	Wild-type	Positive	p.Gly12Cys
3	Wild-type	Positive	p.Gly13Asp
4	Wild-type	Positive	Wild-type
5	Wild-type	Positive	p.Gly12Val
6	Wild-type	Positive	p.Gly12Val
7	Wild-type	Positive	p.Gly13Asp
8	Wild-type	Positive	p.Gly12Asp
9	Wild-type	Positive	p.Gly13Asp
10	Wild-type	Positive	p.Gly13Asp
11	Wild-type	Positive	p.Gly12Val
12	Wild-type	Positive	p.Gly12Asp

4.5 HRM vs DxS Kit

A comparison of *KRAS* mutation results in 115 tumor samples obtained through the DxS Kit and HRM is shown in table 6. In six of the total number of DNA samples analyzed, a consensus results was not obtained. In four of these, a positive result was achieved with the DxS Kit, whereas HRM analysis considered them negative. In the other two samples a positive result was obtained with HRM and considered wild-type using the DxS Kit. The two samples that were not possible to analyze with the DxS Kit were both classified as negative by HRM analysis.

Table 6 – Comparison between HRM and DxS Kit results.

Result	HRM	DxS Kit
Wild-type	65	63
Positive	48	50

In the six samples in which a consensus result was not obtained between the DxS Kit and HRM, sequencing of the HRM products was performed. The two samples negative for mutations with the DxS Kit and positive with HRM had a mutation in either codon 12 or 13, indicating two possible false negative results of the DxS Kit. In the four samples that were positive using DxS Kit and wild-type when analyzed by HRM, a mutation was observed in two of them, whereas in the other two it was not possible to observe a mutation (table 7).

Table 7 – Comparison of results between HRM, DxS Kit and sequencing of HRM products in the six non-concordant samples.

Case Nº	DxS Kit	HRM	HRM sequencing
13	p.Gly12Val	Wild-type	Wild-type
14	p.Gly13Asp	Wild-type	p.Gly13Asp
15	p.Gly12Asp	Wild-type	Wild-type
16	p.Gly12Asp	Wild-type	p.Gly12Asp
17	Wild-type	Positive	p.Gly12Asp
18	Wild-type	Positive	p.Gly13Asp

4.6 SNaPshot

SNaPshot was performed in the 18 samples where a consensual result was not possible to obtain between the two different methodologies previously used. In the 12 samples with a discrepant result between DNA sequencing and HRM, SNaPshot results were concordant with those of sequencing of HRM products and confirmed that 11 of those samples had a mutation and one was wild-type. This indicates the possible presence of 11 false negatives by DNA sequencing and one false positive by HRM before sequencing of HRM products. All six samples with a non-consensual result between HRM and the DxS Kit were positive in the SNaPshot analysis, indicating the presence of 4 false negatives by HRM and 2 false negatives with the DxS Kit (table 8).

Table 8 – Comparison of results between DNA sequencing, DxS Kit, HRM and SNaPshot in the 18 non-concordant samples.

Case Nº	DNA sequencing	DxS Kit	HRM	SNaPshot
1	Wild-type	-	Positive	p.Gly12Asp
2	Wild-type	-	Positive	p.Gly12Cys
3	Wild-type	-	Positive	p.Gly13Asp
4	Wild-type	-	Positive	Wild-type
5	Wild-type	-	Positive	p.Gly12Val
6	Wild-type	-	Positive	p.Gly12Val
7	Wild-type	-	Positive	p.Gly13Asp
8	Wild-type	-	Positive	p.Gly12Asp
9	Wild-type	-	Positive	p.Gly13Asp
10	Wild-type	-	Positive	p.Gly13Asp
11	Wild-type	-	Positive	p.Gly12Val
12	Wild-type	-	Positive	p.Gly12Asp
13	-	p.Gly12Val	Wild-type	p.Gly12Val
14	-	p.Gly13Asp	Wild-type	p.Gly13Asp
15	-	p.Gly12Asp	Wild-type	p.Gly12Asp
16	-	p.Gly12Asp	Wild-type	p.Gly12Asp
17	-	Wild-type	Positive	p.Gly12Asp
18	-	Wild-type	Positive	p.Gly13Asp

4.7 Combined results

Using the combined data from DNA sequencing, the DxS Kit, HRM and SNaPshot, it was possible to establish a consensus result for the samples with non-concordant data when two out of the three methodologies used for the same sample had a similar result. The number of consensus mutations was 77 (41.62%) in the 185 samples analyzed by DNA sequencing, 52 (45.21%) in the 115 samples analyzed with the DxS Kit and 129 (43%) in the total number of samples analyzed. To make an estimate of the sensibility of each methodology for *KRAS* mutation detection, the percentage of consensus mutations was determined for each of them. This was done by dividing the number of mutations detected by each methodology by the number of consensus mutations in the same samples, the results obtained being 85.71% for DNA sequencing, 96.15% for the DxS Kit and 96.90% for HRM (combined result of the 300 samples) (table 9).

Table 9 – Mutation detection frequencies for DNA sequencing, the DxS Kit and HRM

	DNA sequencing (n=185)	DxS Kit (n=115)	HRM		
			(n=185)	(n=115)	(n=300)
Mutations detected/consensus mutations	66/77	50/52	77*/77	48/52	125/129
% of consensus	85.71	96.15	100	92.3	96.90

*One sample was considered positive in the melting curve but negative on HRM product sequencing

V. Discussion

The search for the best methodology for *KRAS* mutation detection is highly relevant because treatment decisions are made based on this genetic alteration. A patient misclassified as wild-type can possibly receive either cetuximab or panitumumab and not benefit from the treatment. If the patient had been correctly classified, he could receive an alternative treatment that would spare him eventual side effects of a drug that is not effective for his cancer and also spare the health care system from further economical burden, as these new biological drugs tend to be very expensive.

This study was designed to evaluate the efficacy of HRM for *KRAS* mutation detection in a clinical setting in comparison to DNA sequencing and the DxS Kit, the two current methodologies used in our laboratory. In order to accomplish this, 300 consecutive CRC tumor samples referred to the Genetics Department of IPO Porto were analyzed with DNA sequencing or the DxS Kit and later by HRM. The frequency of *KRAS* mutations in our study was similar to that described in the literature, which varies from 30 to 50% (Calistri *et al.*, 2005). After a consensus result was achieved for all 300 samples, a mutation frequency of 43% (129 samples) was observed. An estimate of the sensitivity of each methodology was performed using the number of mutations detected by each methodology and the number of consensus mutations, and % of consensus mutation detection of 85.71% for DNA sequencing, 96.15% for the DxS Kit and 96.90% for HRM were observed.

DNA quality is one of the most important factors when performing DNA mutation assays in FFPE tissue. Formalin reduces DNA quality, so the DNA extraction methodology plays a major role. The methodology used in our study to extract DNA was phenol-chloroform, which provides a relatively well purified DNA, albeit in smaller concentrations when compared to traditional methods of DNA extraction. In our study, two samples could not be analyzed by DNA sequencing (table 1) and two other by the DxS Kit (table 2). All these four samples were however analyzable using HRM, which suggests that this methodology is less dependent on DNA quality.

When the findings of the 185 samples analyzed by DNA sequencing were compared with those of HRM, 12 samples with a non-consensus result were found. All of them were classified as wild-type by DNA sequencing and positive with HRM (table 4). Sequencing of the HRM products and SNapShot were performed in these 12 samples and the results indicated that 11 of them were indeed positive for a mutation in codon 12 or 13 of *KRAS* (cases nº1-3 and 5-12). These results are in agreement with the literature that indicates that DNA sequencing is not the most sensitive method for detection of somatic mutations

in tumor samples. For reliable mutation detection by DNA sequencing in these samples, at least 20% of the cells need to have the mutation (Ogino *et al.*, 2005). On the other hand, case nº4 had a small amplitude of the melting curve that was indicative of a mutation present in a minute percentage of the cells in the sample. However, when the HRM product of this sample was sequenced and SNapShot performed, no mutation was detected (table 5 and 8). This result highlights the importance of using another methodology in HRM positive samples, as it serves not only for detection of the specific mutation but also for confirmation of the HRM result.

The DxS Kit tests for the seven most common mutations in codons 12 and 13 of *KRAS*. Although these make for the large part of mutations that are found in mCRC, there are other mutations described that the DxS Kit is unable to detect. In our series of 185 samples that were analyzed by DNA sequencing, two samples with a mutation were detected that would have been reported as mutation negative if the DxS Kit had been used (p.Gly12Phen and p.Gly13Cys) (table 1). In the 115 samples analyzed with the DxS Kit and HRM no such mutations were found, as the only two samples with a mutation that was not detected with the DxS Kit had one of the seven tested by this kit. These two samples that were considered as wild-type when the DxS Kit was used (case nº 17 and 18, table 7), were positive when analyzed with HRM. When the HRM products were sequenced and SNaPshot was performed in these cases, the mutations p.Gly12Asp and p.Gly13Asp were detected. DNA quality of these samples was poor and, although the analysis with the DxS Kit was validated by the software, the DNA quality control of these samples amplified after the 29th cycle (31st and 36th, respectively). In order to guarantee that mutations present in 1% of the cells in a background of wild-type cells are detected, an amplification of the control before the 29th cycle is required as indicated by the manufacturer. Therefore, suboptimal DNA quality could be the reason why the DxS Kit did not detect mutations in these two samples.

Four samples were classified as mutation positive with the DxS Kit and wild-type by HRM analysis. Two of these cases had a very small peak in the electropherogram indicative of a mutation, but in the other two no mutation was observed (table 5). When SNaPshot was performed in these four samples, a mutation was observed in all of them and the type of mutation was concordant with that found by the DxS Kit. This indicates that HRM analysis failed to detect mutations in four of the 115 samples. The DxS Kit is able to detect a mutation present in 1% of the cells in a background of wild-type, cells whereas for detection of mutations with HRM at least 3% of mutated cells need to be

present in the sample (Simi *et al.*, 2008). However, it remains to be demonstrated that a *KRAS* mutation present in less than 3% of the tumor sample is clinically relevant.

One of the main disadvantages of HRM is the fact that is does not provide information about the specific mutation present in the amplicon. Although different mutations in the same amplicon provide different melting curves and it can be possible to differentiate between them when genomic DNA is used, this is harder when tissue samples are being analyzed. The melting curve of an amplicon is defined by its sequence and by the number of mutant sequences in it. The sequence defines the shape of the curve and the number of mutant sequences influences the amplitude of the melting curve. In a subset of samples with different mutations and different percentages of mutant cells in it, differentiation between the different mutations may be very hard to achieve. Therefore, whereas samples with wild-type *KRAS* are relatively well identified, in positive samples one needs to use another method after HRM to be able to specify reliably the mutation present in the sample, for instance by sequencing the HRM amplification products.

After a consensus result was obtained for the 300 samples analyzed in our study, including the SNaPshot analysis in discrepant cases, a comparison between DNA sequencing, the DxS Kit and HRM was possible (table 9). Taking in account the number of mutations detected by each methodology and the number of consensus mutations, sensitivity values of 85.71% for DNA sequencing, 96.15% for the DxS Kit and 96.90% for HRM were observed. This indicates that, although all methodologies detect the majority of mutations, DNA sequencing has the lowest sensitivity and that the performances of HRM and the DxS Kit do not differ significantly. Therefore, both the DxS Kit and HRM are recommended to be used for analysis of *KRAS* mutation *status*. The decision on which methodology to use depends on the equipment available in the laboratory and the reagent costs. The DxS Kit, which has been approved by the FDA, has a cost per sample of about 130€, whereas the cost per sample of HRM is only about 11€, already taking into account that samples are run in duplicate and that DNA sequencing of the product will be needed in 40% of the samples that are positive. Hence, HRM analysis is much more cost-effective than the DxS Kit. Since the equipment requirement for both methodologies is similar, one might consider that, after careful in-lab validation, HRM is a good alternative to the DxS Kit for *KRAS* mutation testing.

When this study started, no reports existed in the literature comparing systematically different methodologies for *KRAS* mutation detection in FFPE samples. Recently, one study evaluated five different methodologies (DNA sequencing, single strand conformation

polymorphism, HRM, TIB Molbiol kit and DxS Kit) using 74 FFPE tumor samples and 80 frozen tumor samples. A consensus of 96% in *KRAS* mutation detection was obtained when the four best performing assays were considered (Whitehall *et al.*, 2009), illustrating the difficulty to establish a gold standard for detection of this type of mutations, as a 100% consensus between the methodologies tested was not obtained. This study also shows that DNA sequencing was the least sensible methodology, detecting only 23 out of the 27 (90.1%) consensus mutations in FFPE samples. Both HRM and the DxS Kit detected all 27 consensus mutations, being the methodologies with a higher sensitivity after exclusion of the TIB Molbiol Kit due to a very high number of false positives. Although this study supports our findings in general, our series of patients is much larger and therefore more closely represents the clinical setting of *KRAS* mutation testing in mCRC.

SNaPshot was used in our study in the 18 samples with discordant findings in order to obtain a consensus result. This recent methodology is also highly sensitive, being able to detect a mutation present in 5% or less of the cells in a sample (Hurst *et al.*, 2009). It is also relatively inexpensive, around 13€ per sample, and it allows the straightforward identification of the various mutations. Our preliminary findings with this methodology are very promising, as it was possible to detect mutations in samples in which HRM or the DxS Kit failed. Further comparative studies are warranted to fully evaluate the role of the SNaPshot methodology in *KRAS* mutation testing in the clinical setting.

VI. Conclusions

Taking in consideration the results obtained in our study, we can conclude that:

- *KRAS* exon 2 mutation frequency in Portuguese patients with mCRC is 43%, being in agreement with the literature in other countries;
- HRM is less dependent on sample DNA quality than the other methods;
- DNA sequencing is the least sensitive methodology for *KRAS* mutation detection among the methodologies tested;
- The DxS Kit and HRM have similar sensitivities for detection of *KRAS* mutations;
- HRM is more cost-effective when compared with the DxS Kit.

VII. Bibliography

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