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Pedro Ribeiro Queirós

Full coding sequence mutation screening of KRAS gene in gastric cancer

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Full coding sequence mutation screening of KRAS gene in gastric cancer

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KRAS MUTATIONS IN MICROSATELLITE INSTABLE GASTRIC TUMOURS: IMPACT OF TARGETED TREATMENT AND INTRATUMOURAL HETEROGENEITY

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Abstract: In gastric cancer (GC), epidermal growth factor receptor (EGFR) overexpression associates with poor prognosis. Addition of a chimeric monoclonal antibody against EGFR (cetuximab) to first-line treatment of metastatic colorectal tumours improved outcomes of patients (stratified for *KRAS* wild-type cancers), whereas GC patients did not benefit from this approach. In GC, however, stratification based on *KRAS* mutations was not performed, and the 30% *KRAS* mutation frequency in Microsatellite instable cancers (MSI), which represents ~4% of total GC was disregarded. Further, intratumoural heterogeneity regarding *KRAS* mutant subpopulations might also contribute to anti-EGFR therapy failure. We assessed the mutational status of the *KRAS* entire coding sequence in 19 MSI-GC cases by multiplex PCR/Sequencing, and used peak height ratio determined from electropherograms from *KRAS* heterozygous mutants and histopathological evaluation to infer tumour heterogeneity in GC. Using 2 multiplex reactions per sample we found that 26% (5/19) of MSI-GC cases harboured *KRAS* mutations (2 G12D, 2 G13D, 1 G12V). No mutations were found outside the codon 12 and 13 hotspots. Our analysis supported the co-existence of *KRAS*-positive and -negative tumour populations in 4/5 MSI-GC cases. In conclusion, the method developed stands as a cost-effective and practical way for mutation screening of the entire *KRAS* coding sequence. *KRAS* mutations are frequent in our series of MSI cases, and are often found in a subpopulation of the tumour and not in the whole tumour. Further studies are needed to access the implications of this heterogeneity in *KRAS* mutant and wild-type tumour clones in anti-EGFR therapy response.

Keywords (4-6): KRAS, microsatellite instability, MSI, gastric cancer, gastric cancer treatment

Introduction

In 2012, gastric cancer's (GC) incidence was reported to be of 952,000 cases (representing 6.8% of total), with a mortality of 723,000 patients (8.8% of total) worldwide. Even though over 70% of cases occur in developing countries, it ranks sixth in developed countries' numbers of both incidence and mortality [1]. Both incidence and mortality have been declining appreciably over the last decades, but they remain high mainly due to advanced stage of disease at presentation, lack of understanding of the complex biology, and morphologic and molecular heterogeneity.

The progress in GC therapy has been limited. Currently, surgery is the only method capable of curing patients and remains the first choice for early-stage disease, although endoscopic resection is being increasingly used in selected early-stage tumours [2]. Adjuvant chemo and radiotherapy may result in improved survival for some of these early-stage patients, but neither the methods nor the outcomes of this approach have been consensual in previous reports [2]. As for advanced-stage GC, chemotherapy should be considered for all patients, given its proven benefits in survival and quality of life, with patients with HER2+ tumours also meriting targeted therapy such as trastuzumab [3]. However, the prognosis remains very poor for patients with GC, with 5-year survival rates of only 30% in Western countries [3]. Thus, the need to better comprehend the molecular behaviour of the disease is urgent, and the current paradigm begets the need of a more appropriate and individualized therapy [2,4].

It has been accepted for many years that there are at least two mutually exclusive molecular types of GC: the chromosomal instability (CIN) type, characterized by structural alterations, namely large deletions, amplifications and translocations; and the microsatellite instability (MSI) type, with instability at the nucleotide level. The MSI type is characterized by alterations in the length of nucleotide repeat sequences, and marks an underlying mismatch repair (MMR) deficiency, meaning that cells fail to recognize errors introduced during DNA replication, which are prone to occur at those nucleotide repeat sequences (microsatellites) [5].

MSI is observed in approximately 15-20% of sporadic GC and also in sporadic colorectal cancer (CRC) with a similar frequency, appearing to be associated with a more favourable prognosis in both cancer types [6,7]. MSI tumours from both colon and stomach are associated with specific profiles of target gene mutations that include insertions/deletions at microsatellite stretches in coding and noncoding sequences, but also with hotspot missense mutations at the *KRAS* (stomach and colon) and *BRAF* genes (colon) [8,6,9]. *KRAS*, a molecule of the MAP kinase (MAPK) pathway and a target gene of the MSI molecular phenotype, was reported to have no difference in mutation frequency between MSI and non-MSI (i.e. microsatellite stable (MSS)) CRC cases [10], or to have lower frequency in MSI as compared with MSS tumours [11,9,12]. Interestingly, in GC, *KRAS* mutations are generally restricted to the MSI group of cancers, and have been described in nearly 30% of these cases using specific analysis of *KRAS* exon 2, affecting the hotspot codons 12 and 13 of the gene [9,13]. Aside from these exon 2 hotspots, *KRAS* mutation frequency seems to be lacklustre. Although in GC, the information on *KRAS* mutations outside these hotspots is scarce, 6.5% of a CRC series of 276 cases demonstrated *KRAS* mutations in other codons of the gene [14].

In CRC, the addition of cetuximab (a chimeric monoclonal antibody against the epidermal growth factor receptor (EGFR)) to irinotecan, fluorouracil, and leucovorin, as first-line treatment for metastatic tumours, was shown to reduce the risk of disease progression, and to increase the chance of response in patients with *KRAS*

wild-type (WT) disease [15]. EGFR positivity, determined by immunohistochemistry, is however regarded as a non-relevant predictive marker of response to this targeted therapy [16]. Indeed, anti-EGFR therapy is reserved for *RAS* WT tumours, since CRC bearing mutations of either *KRAS* or *NRAS* are irresponsive to this treatment [16]. These are gain-of-function mutations that activate the EGFR signalling pathway at a downstream location, rendering the antibodies against this molecule useless [17]. Since this targeted therapy is currently used in CRC patients, predictive testing for *RAS* mutational status is needed, generally using a tumour biopsy sample [17]. However, these specimens are often single, and thus represent only a small region of the tumour, limiting the possibility of detecting positive *RAS*-clones at different sites of the tumour [17]; nonetheless, *RAS* mutational screening is definitive to guide anti-EGFR treatment in CRC patients [16].

In GC, EGFR overexpression occurs and is associated with poor prognosis [18]; this finding, together with the observed improved outcomes regarding anti-EGFR therapy in *KRAS* WT metastatic CRC [15], recurrent or metastatic squamous-cell cancer of the head and neck and advanced non-small-cell lung carcinoma, supported the rationale to perform the EXPAND trial in GC [19]. Cetuximab was added to first-line chemotherapy treatment in advanced non-resectable or metastatic GC, but failed to produce improved outcomes. Also, a subpopulation analysis in this trial revealed no association between EGFR positivity (determined by immunohistochemistry) and overall survival in patients treated either with or without cetuximab, suggesting a non-predictive role for EGFR positivity in GC regarding anti-EGFR therapy, similarly to what occurs in CRC. However, the EXPAND trial failed to account for the presence of *KRAS* mutations in at least 4-10% of GC overall [9,13,20]. Taking in consideration the *KRAS* mutation-mediated anti-EGFR resistance in CRC, this fact could explain, to a certain extent, the failure of the EXPAND trial. Therefore, in case anti-EGFR therapy trials are reconsidered in GC, it would be important to use MSI-positivity (which represents roughly 15-20% of all cases), and its accompanying frequency of *KRAS* mutation in hotspot codons 12 and 13, as part of the GC treatment stratification plan. Also, if a more thorough stratification is to be performed, rigorously checking for mutations, both inside and outside the *KRAS* hotspot regions of the gene, could also prove worthy.

Another important issue in therapy response is intratumoural molecular heterogeneity, a feature of malignant neoplasias that experienced competition between different tumour cells (or clones) for survival, leading to the evolutionary selection of different cell kindred and their progeny [21-23]. A study on metastatic CRC, using a highly sensitive picodroplet digital PCR (dPCR), found that some *KRAS* WT cases actually harboured *KRAS* mutations. Moreover, the fraction of *KRAS*-mutated allele, quantified by dPCR, was inversely correlated with anti-EGFR therapy response rate [24]. These authors hypothesised that while the majority of *KRAS* WT tumour cells in these cases would respond to anti-EGFR therapy, tumour subclones bearing *KRAS* mutations would not. These observations could explain why nearly 50% of CRC cases considered *KRAS* WT, and regarded as responders to anti-EGFR therapy, do not benefit from this approach [24]. This same fact might also contribute to explain the failure of the addition of anti-EGFR therapy to first-line chemotherapy in producing improved outcomes in GC.

With this work, we aim to assess the mutational status of the entire coding sequence of *KRAS* in MSI GC cases, and use peak height ratio determined from electropherograms from *KRAS* heterozygous mutants and histopathological evaluation to infer the tumour heterogeneity in GC cases. To achieve these aims, we developed a multiplex PCR reaction for practical and cost-effective mutation screening of the whole *KRAS* coding sequence; we determined the frequency and type of *KRAS* coding sequence mutations in a series of 19

MSI-positive GC cases; and we inferred tumour heterogeneity in mutated cases by comparing *KRAS* WT and mutated allelic fractions, as determined by electropherogram analysis, with the percentage of tumour cells in matched histological slides.

Materials and Methods

DNA Samples and Extraction

DNA from AGS, IPA220 and MKN28 gastric cancer cell lines was used for optimization of the multiplex PCR. The DNA was extracted from in vitro cultured cells using Spin Tissue Mini Kit (Invisiorb®) according to manufacturer's instructions. DNA quantification and purity were determined using a spectrophotometer (Nanodrop®), and aliquots were created by diluting the DNA of each cell line as to create a working solution of 50ng/µl.

Primary gastric cancer DNA samples were obtained from 19 cases of the Tumour Bank from Centro Hospitalar São João and IPATIMUP. Informed consent was obtained from all patients, and the study was approved by the Hospital's Ethics Committee. These tumour samples had been previously characterized for the presence of MSI phenotype using Pentaplex Kit (Roche®). The DNA content of each sample was determined using the method described above, and aliquots were created in the same manner.

Polymerase Chain Reaction & Primers

Primers were designed in intronic regions flanking *KRAS* exons, and were produced by SIGMA® (Table 1). Working solutions for each primer at a concentration of 100 µM were created. QIAGEN® Multiplex PCR Kit was used according to the manufacturer's instructions. Briefly, the Polymerase Chain Reaction (PCR) was conducted using the following ingredients for each sample: 12.5 µl of QIAGEN® Multiplex Master Mix, 1 µl of primer mix (composed of reverse and forward primers for the corresponding exons in equal proportions), 5 µl of QIAGEN® Q-solution, 1 µl of DNA at working solution and 5.5 µl of DNAse and RNAse-free water. The following cycling conditions were used for PCR: 15 min of 95 ºC for enzyme activation, followed by 30 sec at 94 ºC, 90 sec at 62 ºC and 90 sec at 72 ºC for 3 cycles, 30 sec at 95 ºC, 90 sec at 60 ºC and 90 sec at 72 ºC for 3 cycles, 30 sec at 95 ºC, 90 sec at 58 ºC and 90 sec at 72 ºC for 24 cycles and an extension phase of 72 ºC for the final 10 min.

Sequencing Reaction

After the multiplex PCR, product clean-up was achieved by mixing 10 µl of each multiplex product with 1 µl of Exonuclease I (ExoI) and 2 µl of Thermosensitive Alkaline Phosphatase (FastAP™), and exposing the solution to 37 ºC for 15 min and 85 ºC for 15 min. This was performed separately for each sample. Each exon was sequenced independently by using the corresponding primer (shown in bold in Table 1). For each sequencing reaction 2 µl of cleaned-up DNA were mixed with 0.5 µl of sequencing buffer, 0.4 µl of BigDye® terminator (both of Applied Biosystems®), 0.4 µl of primer (at working solution) and 1.7 µl of DNAse and RNAse-free water. The cycling conditions used for the reaction were the following: 96 ºC for 2 min, followed by 30 cycles of 30 sec at 96 ºC, 15 sec at 54 ºC and 150 sec at 60 ºC; the final extension was performed at 60 ºC for 10 min.

Post-sequencing purification was performed by cross-link dextran gel filtration. A tube for each exon from all the samples was centrifuged at 2000 x g for 4 min with a Sephadex® solution at a concentration of 0.66 mg/ml. The sequencing product was then purified using 5 µl of it under the same centrifugation conditions. The purified product was analysed using an automated sequencer.

Histological & Electropherogram Analysis

Frozen tumour fragments, deposited at the Tumour Bank from Centro Hospitalar São João and IPATIMUP, used for DNA sampling were matched with fragments that were paraffin embedded for histopathological analysis. Haematoxylin and eosin (H&E) stained slides from *KRAS* mutant GC cases were examined by a pathologist from the Department of Pathology of Centro Hospitalar São João (Fátima Carneiro), who determined the approximate percentage of tumour cells present in each slide. The minimal percentage of tumour cells in each case was registered. Then, the (lowest) *expected* mutant-to-WT allelic ratio was determined, that is, the ratio when considering that the whole tumour cell population is mutated for *KRAS*, and that all tumour cells are heterozygous for the mutation (meaning that each cell bears one WT and one mutated allele). The following formula was used (in percentage):

$$\frac{{}^{P}/_{2}}{{}^{P}/_{2} + (100 - P)} \times 100$$

Where P represents the approximate percentage of tumour cells in the slide. With the assumptions made above, ${}^{P}/_{2}$ should represent both the mutated and WT allelic fraction of the tumour cell population, while $(100 - P)$ should represent the WT allelic fraction of non-tumoural cells in the slide.

For each sample submitted to the automated sequencer, an electropherogram was obtained. Peaks on the electropherogram were manually analysed for alterations in the WT sequence of each exon, and altered cases were classified as mutated. For the mutated cases, the peak height for each nucleotide variant was determined using Photoshop® (CC version, Adobe), and the *observed* mutant-to-WT allelic ratio (in percentage) was determined by the following formula:

$$\left(\frac{H_1}{H_2}\right) \times 100$$

Where H_1 represents the height of the peak for the mutated nucleotide, and H_2 represents the height of the peak for the WT nucleotide. This followed the strategy described by Jiang *et al* [25] (Figure 1a). The electropherogram from a common *KRAS* single nucleotide polymorphism (SNP) rs1137282 found in 6/19 GC cases studied was used as a control to determine the expected peak height ratio of the nucleotides (representing each allele) from a constitutional SNP (Figure 1b). The allelic ratios obtained for this SNP in three different GC cases were 89%, 120% and 100%. These were therefore confirmed to represent constitutional heterozygous states.

Results

Full coding sequence mutation screening of KRAS with two PCR reactions

We started by the optimization of a multiplex PCR approach to amplify the full coding sequence of *KRAS*, from exon 2 to exon 6, using three GC cell lines. Following a PCR reaction with 5 primer-pairs, products

were confirmed by agarose gel electrophoresis. Sanger sequencing and electropherogram analysis confirmed successful concomitant amplification and specificity for exons 2 to 5, but not for exon 6. Exon 6 was therefore amplified and sequenced independently (Figure 2).

The same amplification protocol was then applied to 19 GC tumour samples. Agarose gel electrophoresis confirmed the amplification reaction, and sequencing reactions were successful for all cases (Supplementary Table 1).

26% of MSI GC cases harbour mutations in the KRAS coding sequence

Confirming the feasibility of the amplification protocol, we verified that, as expected, the AGS cell line presented a *KRAS* mutation in exon 2 (G12D), while the other two cell lines were WT for all exons. From the MSI-positive GC cases studied, 5/19 (26%) were found to be mutated. Two cases carried a G13D mutation, two cases carried the G12D mutation and one case carried the G12V mutation. A common SNP (rs1137282) was also identified in 7 GC cases (6 in a heterozygous and 1 in a homozygous state), but no mutations were identified outside the hotspot region of the gene (Table 2, Supplementary Table 1).

Histological and electropherogram analysis of KRAS mutated cases allow inferring the presence of tumour heterogeneity

The electropherogram analysis from *KRAS* mutated cases indicated that the peak height corresponding to the mutant allele was variable among GC cases (Figure 3). This could have occurred either due to variable degrees of stromal contamination in different cases, or due to molecular intratumoural heterogeneity (presence of both *KRAS* mutant and *KRAS* WT subpopulations in the same GC tumour). To address this issue, we took advantage from the fact that the frozen fragment used for DNA extraction had a twin fragment that had been paraffin embedded. We therefore re-evaluated the H&E slides of each *KRAS* mutated case to assess the effective percentage of tumour cells in each fragment (Table 3). For each case, the percentage of tumour cells was registered for comparison with the allelic ratio observed in the corresponding electropherogram. Figure 3 shows examples of light microscopy images from the mutated cases and the corresponding electropherogram peaks. Comparing the expected mutant-to-WT allelic ratio obtained from the histopathological analysis with the peak height ratio determined by electropherogram analysis (observed ratio), 4/5 cases demonstrated an “observed ratio” lower than the “expected ratio” (Table 3). Overall, the concordance between observed and expected ratios was low, suggesting the existence of heterogeneity in tumour populations in 4/5 cases, where some are positive for *KRAS* mutations and others are negative for these mutations.

Discussion

With this work we intended to develop a multiplex PCR approach that could be used to screen the full coding sequence of *KRAS* using low amounts of DNA in a cost effective way, and use the sequencing analysis in *KRAS* mutant cases, as well as corresponding histological analysis, to infer molecular heterogeneity related with *KRAS* mutant cancer cell populations. This information may be important if anti-EGFR treatment is re-evaluated in GC, to allow correct stratification of cases for this type of targeted therapy.

Using a multiplex PCR reaction for exons 2 to 5 and an independent PCR reaction for exon 6, mutational analysis of the entire coding sequence of the *KRAS* gene was achieved for all the 19 MSI positive GC cases analysed. Reducing the number of PCR reactions needed from a total of 6 (one per exon) to only 2 makes the method developed in this study an efficient, yet cost-effective and practical way of amplifying the entire coding sequence of this gene. Also, this was achieved using low quantities of DNA. At the moment, this is a relevant issue in CRC, as the use of neoadjuvant therapy in these patients is likely to increase in the future. In that case, the only tissue available for predictive testing will be the diagnostic biopsy samples, used to confirm the presence of the malignant tumour, which may not be present in abundant quantities. Thus, if *KRAS* testing is to be applied in a predictive fashion for neoadjuvant treatment, mutational analysis in limited quantities of DNA could be a problem, suggesting the need for strategies that make the most out of the available material, whilst not demanding state of the art technology or high budget, like the one designed in this study.

The frequency of *KRAS* mutations encountered (approximately 26%) is in accordance with the frequency described in previous reports [9,13]. No mutations were found outside the hotspot region of the gene, which is also in accordance with some previous studies [20]. Even though in the present study no mutations were found outside the hotspots, studies addressing the efficacy of anti-EGFR therapy in GC should not neglect the small percentage of cases that might be mutated outside the hotspot region, since they contribute to therapeutic failure. In fact, while we were developing this work, the TCGA Consortium reported the whole Exome Sequencing analysis of almost 300 GC cases and revealed that in MSI GC cases, the frequency of *KRAS* mutations was of 23%, which matches our own findings [20,26,27]. That study also found that MSS GC cases had a *KRAS* mutation frequency of 6%. One of the premises of the present work was that *KRAS* mutations were restricted to MSI GC cases. The premise of most previous studies was that KRAS mutations only occurred in hotspot codons 12 and 13, and therefore only exon 2 was screened [9,13]. The recent discovery of *KRAS* mutations in 6% of MSS GC cases is due to a more thorough search for mutations in the whole gene, considering both hotspot and non-hotspot regions, highlighting the pertinence of a strategy for *KRAS* mutation screening, that should now be extended also to MSS GC cases. An additional finding of the TCGA Consortium was the amplification of *KRAS* gene in approximately 7% of GC cases overall [26,27]. In CRC, *KRAS* amplification is also found, although to a much lesser degree (<1%, according to one study) [28], and it has been postulated that it might be responsible for resistance to anti-EGFR therapy in some cases.

Taking in consideration the findings and the experience in CRC, namely regarding the *KRAS*-related resistance to anti-EGFR therapy, it is possible that the failure of this targeted therapy in GC is actually justified by the frequency of *KRAS* alterations. In summary, if anti-EGFR therapy is ever reconsidered in GC, patient stratification based on all these mechanisms and disease subsets needs to be considered.

A perspective that one should not disregard when stratifying patients for treatment is that tumours are heterogeneous and most often bear a fraction that will respond to a given targeted treatment, and a fraction that will not. If screening strategies are sufficiently sensitive to identify a fraction of the tumour that is likely to respond, should these tumours be treated or not?

In CRC, tumour heterogeneity for *KRAS* genotype has been well documented [24,29,30] and the fraction of mutated cells in the tumours is variable [31]. However, the real impact of this in the behaviour of the tumour as a unit is not known, nor are the implications in survival, response to therapy and other outcomes. To determine this, studies that include sensitive assays like the one used by Laurent-Puig *et al* [24] in search of a

subpopulation of *KRAS* mutant clones in *KRAS* WT cases, the use of these findings for patient stratification regarding anti-EGFR therapy, and the evaluation of response to therapy, could shed some light in this matter.

In the present study, we aimed at determining the relative allelic fraction of *KRAS* mutant and WT alleles, while correlating this value with the fraction of tumour cells in a sample. We have shown that 4/5 of the *KRAS* mutated cases of our series present with both *KRAS* WT and *KRAS* mutant clones, and this finding correlates with tumour heterogeneity. Although peak height analysis is a relatively simple technique to determine quantitative allelic variations, it has been used before in a similar fashion [25,32]; This approach has been shown to be highly comparable to more laborious, expensive and time-consuming methodologies such as pyrosequencing and cloning-based sequencing [25]. Therefore, peak height analysis, in worst case scenario, may be a useable semi-quantitative method for evaluating allelic variation. Recent studies have also supported heterogeneity in GC with respect to established (HER2) and potential target genes [33]. Indeed, it stands as highly likely that, in the future, as more and more studies begin to unravel the field of tumoural heterogeneity, that this becomes a basic notion that influences patient stratification and treatment.

If a *KRAS* mutation was to appear in the early stages of neoplastic development, it would make sense that the large majority of the cells harbour it, and this would in turn return a very high observed mutant-to-WT ratio. On the other hand, if mutations in *KRAS* occur as part of the tumour progression, this ratio would decrease. So, assuming that tumours have a tendency for heterogeneity, the tumour fragment taken for DNA extraction and mutation screening should be matched with a fragment that is submitted to histological analysis to increase the likelihood of obtaining reliable result on allelic fractions. To be more precise, the mutation analysis should even be done in material from the slide used for histological analysis and to determine the proportion of tumoural cells. In the present study, we used the first option to *infer* the heterogeneity in the sample, but we believe that future studies should follow the more meticulous approach described in the second option.

In conclusion, the method for determining the full coding sequence of *KRAS* developed in this study stands as an easy, cost-effective and practical way to screen the entire coding sequence of the gene for mutations. The findings regarding mutation frequency suggest that *KRAS* mutation is frequent in MSI-positive GC cases, but if other mechanisms are also screened both in MSI and MSS cases, it is likely that the contribution of *KRAS* will increase in GC overall. Intratumoural heterogeneity and its practical consequences are still a clouded area, and advances in the assays used to determine the mutational status of tumours are bound to allow a deeper understanding of this feature. Also, our analysis for tumoural heterogeneity indicates that some MSI GC patients labelled as mutated for *KRAS* might bear the mutation in only a cellular clone and not the whole tumour. Further studies are needed to access the implications of this heterogeneity in therapy response. If new studies are designed to determine the efficacy of anti-EGFR therapy in GC, these should account for the differences at the molecular and genetic level between MSI and MSS cases, bearing in mind the high prevalence of *KRAS* mutated cases in the MSI group, the possibility for *KRAS* mutations in the MSS group, and the overall prevalence of *KRAS* amplification, with their accompanying anti-EGFR resistance. The mutational status in future studies should also consider using gene-wide assays for *KRAS* such as the one developed in this work, and perhaps for other *RAS* family members (e.g. *NRAS*), which have demonstrated to confer resistance to therapy with anti-EGFR molecules in CRC cases [16].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1 Determination of peak heights. **a**. A sample electropherogram is shown to illustrate how peak height measurements were made. H_1 represents the height of the peak for the mutated nucleotide and H_2 represents the height of the peak for the WT nucleotide. Below the electropherogram the formula used to calculate the allelic ratio is shown **b**. Three electropherograms used as controls and displaying an example of a common *KRAS* SNP found in the same cases. For this SNP, the height of each peak remains the same in each GC case, representing equivalent allelic fractions from both alleles

Fig. 2 *KRAS* amplification strategy. A representative picture of agarose gel electrophoresis for each reaction is shown. On the right, an example of the obtained electropherograms is shown

Fig. 3 Electropherogram and histological analysis of the mutated cases. In each part of the image, the upper left corner shows a 4x magnification light microscopy image of a slide of the tumour sample, reflecting the global aspect of the piece; the lower right corner shows a 20x magnification of a region in the same slide, demonstrating particular features for each case; the lower left corner shows the respective electropherogram and the corresponding mutation. **a.** case number 77. *Upper left corner:* image shows areas of tubular aspect (middle of the image), progressing to solid aspect and a region of fibrosis (upper right corner); *Lower right corner:* a few tubular structures are visible, along with the solid aspect (upper left corner) and a mild lymphoplasmocytic infiltrate. Tumoural cells make up at least 80% of the content. **b.** case number 88. *Upper left corner:* high cellular content is predictable, together with areas of fibrosis; *Lower right corner:* tubular structures are sparse and with low differentiation, and an abundant lymphoplasmocytic infiltrate is present, which accounts for the estimated percentage of tumoural cells of at least 60%. **c.** case number 95. *Upper left corner:* solid appearance with soft basophilic staining and areas of fibrosis; *Lower right corner:* solid tumour with adenosquamous differentiation. Tumoural cell percentage estimated to be at least 60%. **d.** case number 107. *Upper left corner:* some tubular-like structures are visible together with fibrosis; *Lower right corner:* a mix of tubular and isolated cell patterns is observed, with a desmoplastic reaction. Tumoural cells should account for at least 80% of the cellular content. **e.** case number 181. *Upper left corner:* image filled with tubular appearing structures; *Lower right corner:* abundant tubular structures occupy the whole photograph. Tumoural cell percentage estimated to be of at least 90%

Tables

Table 1 Primer Identification, sequence and respective fragment size for each *KRAS* exon to be amplified. Primers used for Sanger sequencing are identified in bold.

PRIMER IDENTIFICATION		PRIMER SEQUENCE	FRAGMENT SIZE
EXON 2	**Forward**	5'-AAAAGGTACTGGTGGAGTATTTGA	293 bp
	Reverse	5'-TCATGAAAATGGTCAGAGAAACC	
EXON 3	Forward	5'-TGCACTGTAATAATCCAGACTGTG	296 bp
	Reverse	5'-TGGCATTAGCAAAGACTCAAAA	
EXON 4	Forward	5'-AGTTGTGGACAGGTTTTGAAAGA	351 bp
	Reverse	5'-ATGCCCTCTCAAGAGACAAAAA	
EXON 5	**Forward**	5'-AAACTTCTTGCACATGGCTTTC	275 bp
	Reverse	5'-GGTTGCCACCTTGTTACCTTTA	
EXON 6	**Forward**	5'-TTCAGTTGCCTGAAGAGAAACA	358 bp
	Reverse	5'-CTGCATGGAGCAGGAAAAA	

Table 2 Mutated cases and their mutated codon, nucleotide and the resulting mutation, expressed as the amino acid change.

MUTATED CASE	MUTATED CODON	NUCLEOTIDE ALTERATION	TYPE OF MUTATION
77	13	GGC to GAC	G13D
88	13	GGC to GAC	G13D
95	12	GGT to GAT	G12D
107	12	GGT to GAT	G12D
181	12	GGT to GTT	G12V

Table 3 Mutated cases and respective percentage of tumoural cells, ratios and predicted heterogeneity.

CASE	% TUMOURAL CELLS (histological)	*EXPECTED* MUT/WT ALLELIC KRAS RATIO	*OBSERVED* MUT/WT ALLELIC KRAS RATIO[a]	PREDICTED HETEROGENEITY
77	≥80%	67%	42%	Yes
88	≥60%	43%	22%	Yes
95	≥60%	43%	60%	No
107	≥80%	67%	48%	Yes
181	≥90%	82%	37%	Yes

[a]This ratio was calculated based on the peak height of mutant and WT alleles for codons 12 and 13 of *KRAS*

Supplementary Table 1 Map of mutations in KRAS coding sequence of the 19 MSI-positive cases. Mutated cases are depicted in red, with the respective aminoacid change. WT cases are represented in green. SNP are shown in yellow.

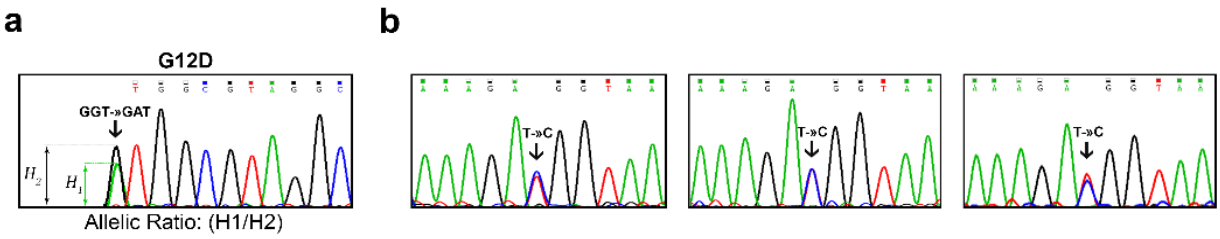

Fig. 1

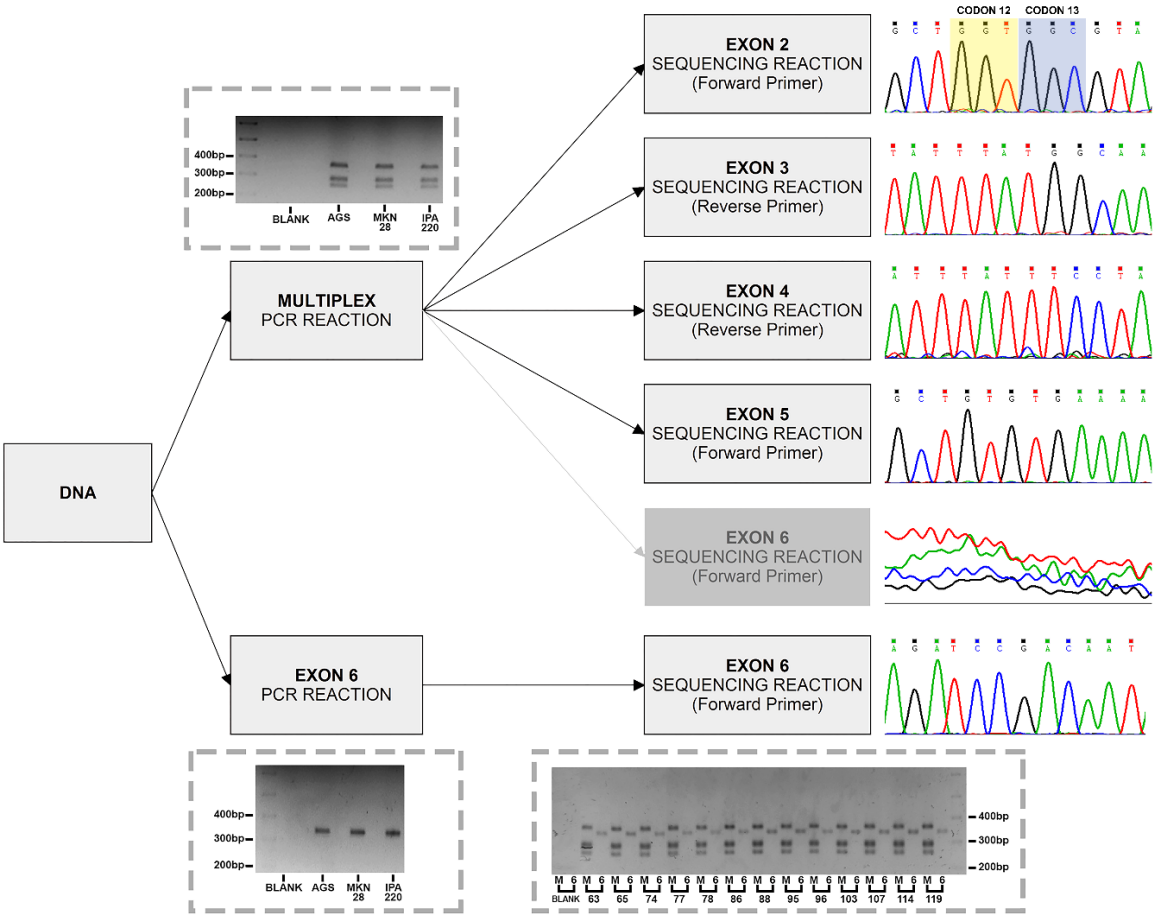
DNA
MULTIPLEX
PCR REACTION
EXON 6
PCR REACTION
EXON 2
SEQUENCING REACTION
(Forward Primer)
EXON 3
SEQUENCING REACTION
(Reverse Primer)
EXON 4
SEQUENCING REACTION
(Reverse Primer)
EXON 5
SEQUENCING REACTION
(Forward Primer)
EXON 6
SEQUENCING REACTION
(Forward Primer)
EXON 6
SEQUENCING REACTION
(Forward Primer)
CODON 12
CODON 13
400bp
300bp
200bp
BLANK
AGS
MKN 28
IPA 220
M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6 M 6
BLANK 63 65 74 77 78 86 88 95 96 103 107 114 119

Fig. 2

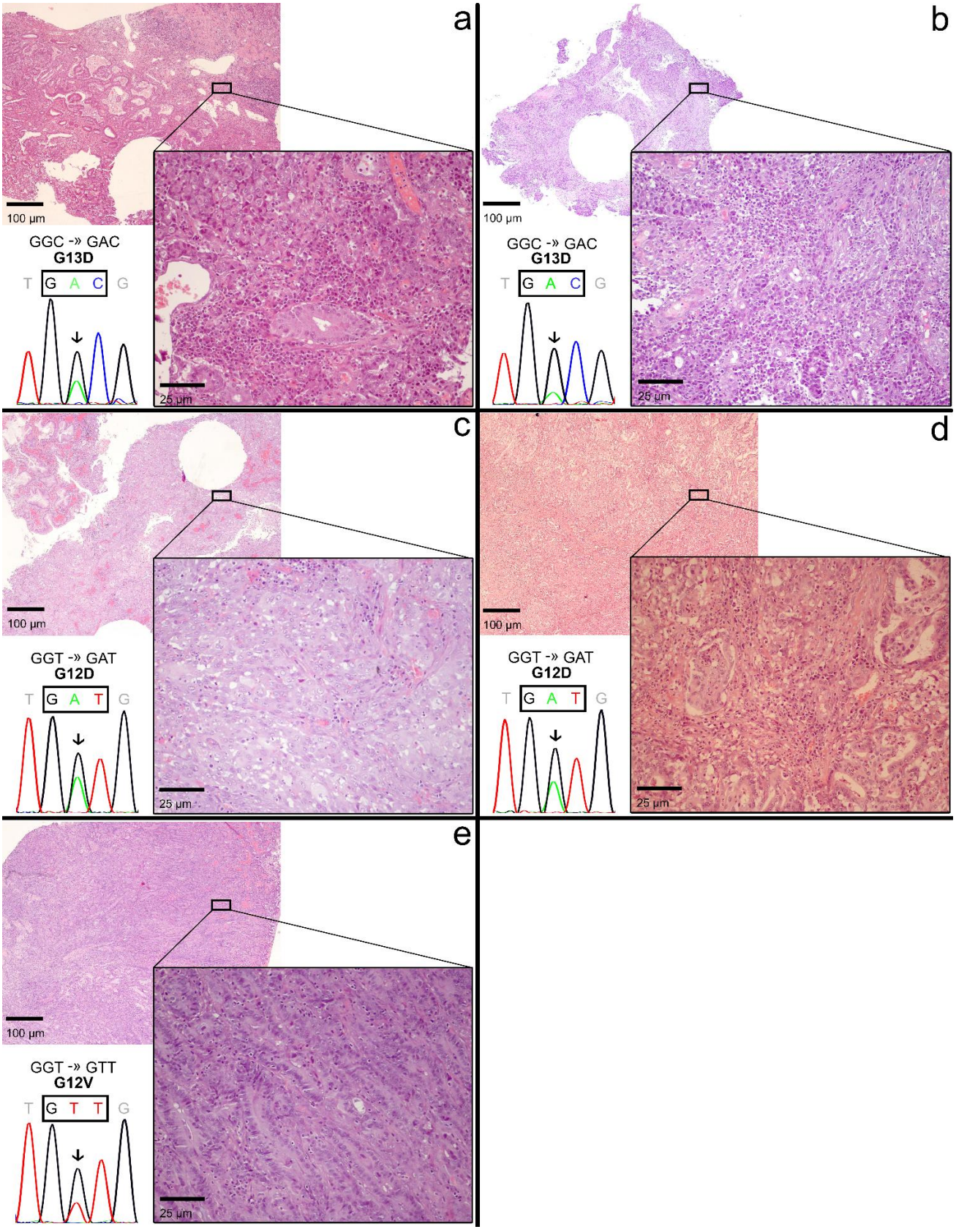
a
100 µm
GGC -» GAC
G13D
T G A C G
25 µm
b
100 µm
GGC -» GAC
G13D
T G A C G
25 µm
c
100 µm
GGT -» GAT
G12D
T G A T G
25 µm
d
100 µm
GGT -» GAT
G12D
T G A T G
25 µm
e
100 µm
GGT -» GTT
G12V
T G T T G
25 µm

Fig. 3

CASE NUMBER	EXON 2	EXON 3	EXON 4	EXON 5	EXON 6
63	WT	WT	WT	WT	rs1137282
65	WT	WT	WT	WT	rs1137282
74	WT	WT	WT	WT	WT
77	G13D	WT	WT	WT	WT
78	WT	WT	WT	WT	rs1137282
86	WT	WT	WT	WT	WT
88	G13D	WT	WT	WT	WT
95	G12D	WT	WT	WT	WT
96	WT	WT	WT	WT	WT
103	WT	WT	WT	WT	WT
107	G12D	WT	WT	WT	rs1137282
114	WT	WT	WT	WT	WT
119	WT	WT	WT	WT	WT
157	WT	WT	WT	WT	WT
175	WT	WT	WT	WT	rs1137282
177	WT	WT	WT	WT	rs1137282
181	G12V	WT	WT	WT	WT
189	WT	WT	WT	WT	rs1137282
191	WT	WT	WT	WT	WT

Supplementary Table 1

Agradecimentos:

À Doutora Carla Oliveira, à Doutora Gabriela Almeida e à Doutora Fátima Carneiro por toda a atenção e tempo dispensado.

A toda a equipa do Expression Regulation In Cancer Group, por me terem ajudado das mais diversas formas.

À Irene Gullo, por me ter ajudado com as preciosas fotografias.

ANEXOS

Instructions for Authors

Manuscript Structure

The following types of articles will be considered for publication:

All manuscripts are subject to copy editing.

- Original articles

  Original articles should not exceed 10 printed pages. This equals about 30 typewritten pages (A4 paper) with 33 lines per page and 65 strokes per line.

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Only invited Editorials, Review articles and Reports on meetings and clinico-pathologic trials will be published. Review articles for Virchows Archiv should comprise no more than 5 printed pages including literature (50- 60 citations). The review should begin with an introduction and end with a conclusion or a perspective. It should be accompanied by a short informative abstract including key words. Four to 5 illustrations are welcome. Please let me know if you need more space for illustrations.

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The title page should include:

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- The e-mail address, telephone and fax numbers of the corresponding author

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

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Please provide 4 to 6 keywords which can be used for indexing purposes.

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- Use the automatic page numbering function to number the pages.
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- LaTeX macro package (zip, 182 kB)

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Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

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Reference citations in the text should be identified by numbers in square brackets. Some examples:
1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
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- Journal article

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  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

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- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

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  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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- All tables are to be numbered using Arabic numerals.

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- For each table, please supply a table caption (title) explaining the components of the table.
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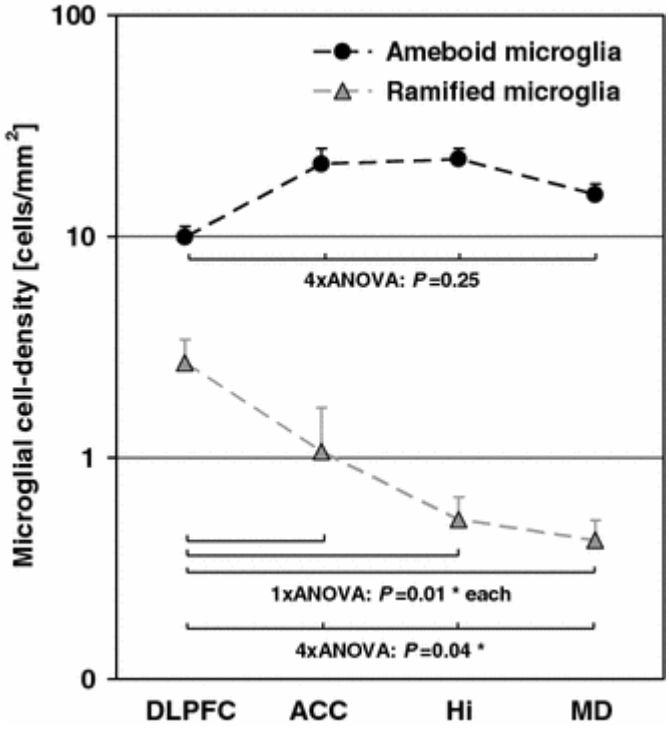

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
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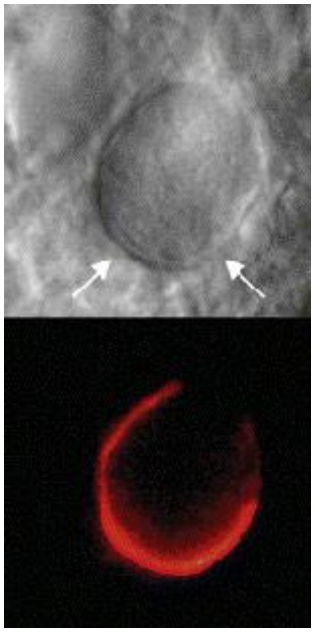

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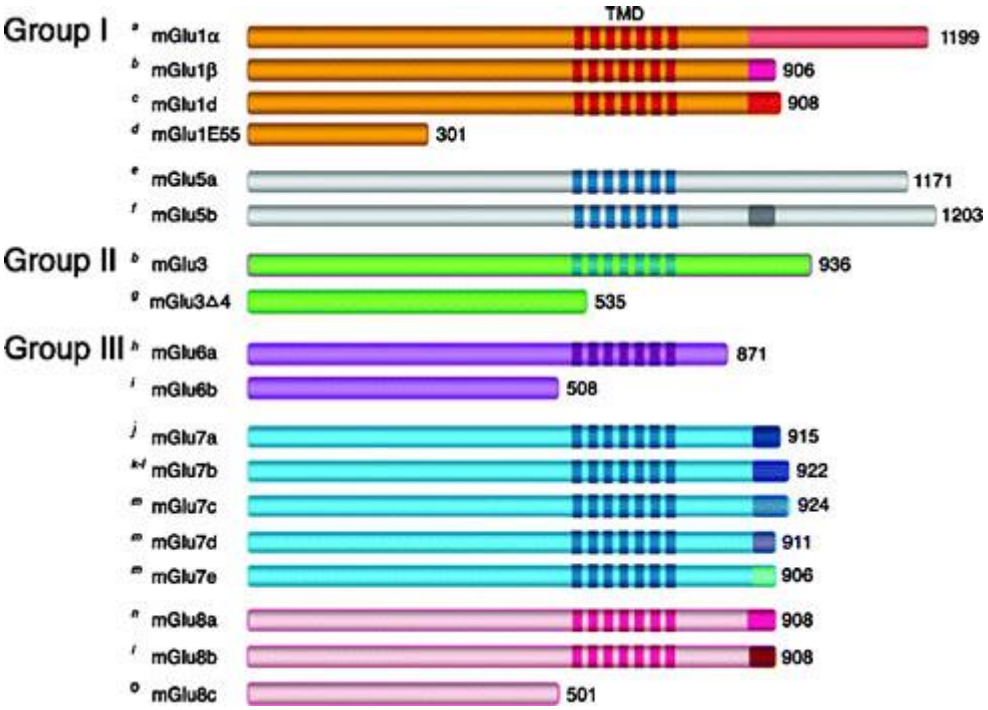

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- Do not include titles or captions within your illustrations.

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- All figures are to be numbered using Arabic numerals.
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AUTORIZADO

CONSELHO DE ADMINISTRAÇÃO @ REUNIÃO DE 9 MAIO 2013

Presidente do Conselho de Administração

(Prof.. Doutor António Ferreira)

Directora Clínica | Enfermeira Directora | Vogal Executiva | Vogal Executivo

(Dra Margarida Tavares) | (Enfermeira Eurídice Pereira) | (Dra. Maria Barros) | (Dr. João Oliveira)

Ao CA c/ proposta
Aprovado DC
7.5.2013

Exmo. Senhor Presidente do Conselho de Administração do Centro Hospitalar de São João (C.H.S.J.),

Assunto: Pedido de autorização para a realização do estudo

Nome do Investigador Principal: Carla Oliveira

Título do projecto de investigação: Using NGS to uncover structural and regulatory variation that dictate gastric cancer stratification

Pretendemos utilizar material do Banco de Tecidos e Tumores do Serviço de Anatomia Patológica do Centro Hospitalar de São João (C.H.S.J.) para a realização do projecto de investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador Principal, a sua autorização para a realização do estudo.

Para o efeito, anexo toda a documentação referida no dossier dessa Comissão respeitante a projectos de investigação.

Com os melhores cumprimentos.

Porto, 22 de Fevereiro de 2013

O INVESTIGADOR PRINCIPAL

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SÃO JOÃO

Comissão de Ética para a Saúde – Centro Hospitalar São João

Parecer

Título do Projecto: Using NGS to uncover structural and regulatory variation that dictate gastric câncer stratification.

Nome do Investigador Principal: Doutora Carla Oliveira

Local onde sera realizado o estudo: IPATIMUP e Serviço de Anatomia Patológica do CHSJ, havendo autorização da respectiva Directora de Serviço para a realização da mesma.

Objectivo do estudo:
"Perform Whole Genome Sequencing (WGS), RNAseq and Bisulphite sequencing of carefully selected groups of gastric câncer (GC) cases, displaying clear molecular phenotypes. The selection criteria of the cases is of paramount importance and draws a clear line between this project and other general initiatives aiming at WGS of this type of neoplasia.
The ultimate goal of this project is to pinpoint relevant genetic alterations and associated biological mechanisms associated with GC. This project will have the long term objective of identifying new therapeutics targets in GC and the selection of new biomarkers that can be used for early diagnosis, prognosis and predicting response to therapy."

Estudo retrospectivo recorrendo a técnicas de sequenciação genetica (WGS, RNAseq & Bisulphite) de casos / grupos selecionados de cancro gástrico, utilizando amostras congeladas de neoplasias gástricas arquivadas no Banco de Tecidos e Tumores do CHSJ.

Período previsto de conclusão: Dezembro 2014

Benefício / Risco: N/A

Respeito pela liberdade e autonomia do sujeito do ensaio: N/A

Confidencialidade dos dados: está garantida a confidencialidade dos dados e esta informação será restrita ao investigador principal.

SÃO JOÃO

A Investigadora Principal dispõe de competência técnica e científica para a realização do estudo.

Custos: O estudo não prevê custos acrescidos para a instituição.

Parecer: Em face da análise do protocolo de estudo, proponho a sua aprovação pela CES do CHSJ.

Porto, CHSJ, 21 de março de 2013

O Relator

Dr. John Preto

CENTRO HOSPITALAR DE **SÃO JOÃO** E.P.E.
PORTO (Sede) Alameda Professor Hernâni Monteiro 4200-319 Porto T + 351 225 512 100 geral@hsjoao.min-saude.pt
VALONGO Rua de Misericórdia 4440-563 Valongo T + 351 224 220 019 www.hsjoao.min-saude.pt

7. SEGURO

a. *Este estudo/projecto de investigação prevê intervenção clínica que implique a existência de um seguro para os participantes?*

SIM ☐ (Se sim, junte, por favor, cópia da Apólice de Seguro respectiva)
NÃO ☐
NÃO APLICÁVEL ☒

8. TERMO DE RESPONSABILIDADE

Eu, **Carla Isabel Gonçalves Oliveira**, abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 25 / Fevereiro / 2013

Carla Oliveira

O Investigador Principal

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO

emitido na reunião plenária da CES de 22 / Fev. / 2013

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética