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ORIGINAL ARTICLE

A subset of colorectal carcinomas express c-KIT protein independently of *BRAF* and/or *KRAS* activation

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Abstract c-KIT is a tyrosine kinase receptor found to be overexpressed in several tumours, namely, GISTs, breast, lung, prostate, ovarian and colorectal carcinomas (CRC). We aimed at determining the frequency of c-KIT expression and mutations in a series of 109 CRC cases (73 primary tumours and 36 lymph node metastases) characterised for *KRAS* and *BRAF* mutations. We also aimed at analysing the cellular effects of STI571/Gleevec in CRC-derived cell lines displaying c-KIT expression and *KRAS* or *BRAF* mutations. By immunohistochemistry, we found c-KIT overexpression in 15% (11/73) of primary tumours and in 14% (5/36) of metastasis; however, cases showing overexpression did not show *c-kit* mutations in *hotspot* regions. The majority (64%) of primary tumours with c-KIT overexpression had mutations at *KRAS–BRAF* genes. The same was true for 60% of the metastases. We treated CRC cell lines with STI571/Gleevec and verified that it inhibits proliferation and induces apoptosis in all cell lines. In conclusion, overexpression of c-KIT is observed in a subset of primary and CRC metastases in the absence of *c-kit* mutations. STI571/Gleevec increases apoptosis in CRC cell lines independently of its genetic profile, suggesting that STI571/Gleevec is likely to be an alternative drug for the clinical trials of CRC.

Keywords Colorectal carcinoma · c-KIT · STI571/Gleevec · *BRAF* · *KRAS*

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Introduction

The proto-oncogene *c-kit* encodes a tyrosine kinase receptor normally expressed in a variety of human tissues including hematopoietic stem cells, melanocytes, mast cells, germ cells and the interstitial cells of Cajal [1]. The ligand for c-KIT is the stem cell factor (SCF) that activates c-KIT tyrosine kinase via homodimerisation and autophosphorylation of the receptor at specific tyrosine residues in the intracellular domain of the receptor [28, 40]. c-KIT activates several signal transduction pathways implicated in cell survival, proliferation, differentiation, adhesion and migration [4, 6].

Aberrant expression of c-KIT has been reported in several tumours, namely, gastrointestinal stromal tumours (GISTs), breast, lung, neural, prostate and ovarian tumours [11, 14, 28, 35]. Gain-of-function mutations of the *c-kit* gene and overexpression of c-KIT have been reported in mast cell tumours [12, 19], GISTs [15, 16, 39], germ cell tumours (GCTs) [32, 36] and melanomas [37, 38]. The five mutational hotspots at the *c-kit* gene have been identified in exons 9, 10, 11, 13 and 17 [27, 31].

In colorectal cancer (CRC), data concerning expression of c-KIT is controversial. Some authors have demonstrated

overexpression of both c-KIT transcripts and protein in CRC [6, 28], while others have failed to detect the c-KIT protein in the majority of CRC examined [20, 26]. In CRC, oncogenic mutations in *KRAS* mutations are well known to occur in the first steps of carcinogenesis both in MSI and MSS settings [22, 23, 25]. In sporadic CRC, oncogenic *BRAF* mutations are found in about 12% of the tumours analysed, mainly in MSI cases (31–45%) [9, 10, 22, 25]. *BRAF* mutations are also very frequent in melanomas [9]. Recently, a subset of melanomas was described to harbour *c-kit* activating mutations associated to c-KIT expression. Moreover, *c-kit*-activating mutations were shown to be alternative to *BRAF* mutations [8, 37, 38]. Further, similarly to *KRAS* and *BRAF*, it has been demonstrated that activated c-KIT signals through the MAPK pathway [9, 29, 33]. The lack of such knowledge in CRC leads us to hypothesise that c-KIT expression/mutation may be alternative to *KRAS* and *BRAF*-activating mutations.

Having available CRC-derived cell lines displaying different genetic background: HCT116 expresses c-KIT and harbours a RAS^{G13D} mutation, CO115 harbours a $BRAF^{V600E}$ mutation and HCT15 harbours a RAS^{G13D} mutation, we study the cellular effects of an inhibitor of c-KIT receptor tyrosine kinase (STI571/ Gleevec). Imatinib mesilate (STI571/Gleevec), an inhibitor of Bcr-Abl and c-Abl tyrosine kinase, primarily designed to treat chronic myeloid leukaemia (Philadelphia chromosome-positive), is also an inhibitor of c-KIT receptor tyrosine kinase and platelet-derived growth factor (PDGF) receptor. This drug is currently the therapeutic choice for metastatic GISTs known to express c-KIT and PDGFR [13, 17, 34]. We studied the cellular effects (proliferation and apoptosis) of STI571/Gleevec in CRC cell lines in an attempt to clarify whether STI571/Gleevec may be useful for treating CRC patients harbouring different genetic alterations.

Materials and methods

Tumour specimens

A total of 109 formalin-fixed paraffin-embedded samples (73 primary sporadic colorectal carcinomas, 36 of which with a corresponding lymph node metastasis) were obtained from S. João Hospital, Porto, Portugal. Age of the patients, location of the tumour, histological classification, presence or absence of lymph nodes metastasis, Dukes classification and TNM tumour staging were available from every case.

Immunohistochemical study

Immunohistochemistry was performed using the avidin-biotin-peroxidase method. Briefly, endogenous peroxidase activity and non-specific binding were blocked by hydrogen peroxide and a blocking reagent in the LSAB2 kit (Dako), respectively. The slides were incubated overnight at 4°C using a dilution of 1/500 with the primary antibody and then incubated sequentially at room temperature with a biotinylated secondary antibody for 30 min, streptavidin peroxidase for 15 min and Dako DAB for 10 min. The slides were then counterstained with Mayer's hematoxylin and amoniacal water. We used as primary antibodies rabbit polyclonal antibody raised against human c-KIT (CD117, Dako) and as a negative control, a rabbit polyclonal antibody to single-stranded DNA (ssDNA; Dako, Glostrup, Denmark). We used as a positive control a case of GIST with *c-KIT* expression and as internal positive control, interstitial cells of Cajal [30]. Membrane and/or cytoplasmic immunoreactivity for CD117 was considered positive.

Genomic DNA extraction and mutational analysis of *c-kit*

c-kit mutation screening was restricted to cases displaying c-KIT immunoexpression. Genomic DNA was extracted from dissected paraffin-embedded material using phenol-chloroform according to standard procedures. The fragment encompassing exon 9, 10, 11, 13 and 17 was amplified by polymerase chain reaction (PCR) in all carcinoma samples. Genomic DNA (25–100 ng) was amplified by PCR using the following cycling conditions: 30 s at 94°C, 30 s at 58°C and 45 s at 72°C for 40 cycles. All PCR products were purified and directed sequenced on an ABI Prism 377 Automatic sequencer (Perkin-Elmer, Foster City, CA) using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). All sequencing reactions were performed in both forward and reverse directions, and all mutations were confirmed by an independent PCR amplification. For amplification of exon 9, 10, 11, 13 and 17 of *c-kit* gene, the following primers were used: Ex9F: 5′-GAT GCT CTG CTT CTG TAC TG-3′; Ex9R: 5′-AAG GGG ATG TTT AGG CTC TG-3′); Ex10F: 5′-TCC TGC CAA AGT TTG TGA TTC-3′; Ex10R: 5′-CCA GAG TGC TCT AAT GAC TG-3′); Ex11F: 5′-CCA GAG TGC TCT AAT GAC TG-3′; Ex11R: 5′-TGG TCA GTA TGA AAC AGG GG-3′); Ex13F: 5′-CAT CAG TTT GCC AGT TGT GC-3′; Ex13R: 5′-CGT GTC CAA GCT GCC TTT TA-3′); Ex17F: 5′-CTT TTC TCC TCC AAC CTA ATA G-3′; Ex17R: 5′-CCC ATT CTC TGC TTG ACA GT-3′).

KRAS and *BRAF* and mutation screening

KRAS and *BRAF* mutations were screened in all 109 CRC cases (primary tumours and metastases). In all cases, we analysed the mutation status of *KRAS* by direct sequencing. Mutational analysis of *BRAF* was performed by single-

stranded conformation polymorphism and heteroduplex analysis (SSCP/HA) [23].

CRC Cell lines, culture conditions and drug treatment

Three CRC cancer cell lines were analysed (CO115, HCT116 and HCT15). HCT116 express c-kit and harbours a $KRAS^{G13D}$ mutation, CO115 harbours a $BRAF^{V600E}$ mutation and HCT15 harbours a $KRAS^{G13D}$ mutation. The human colon carcinoma-derived cell lines CO115, HCT116 and HCT15 were grown in RPMI 160 medium (with L-glutamine and HEPES—Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen) and 100 units/ml penicillin-streptomycin (Gibco, Invitrogen). All cells were grown in a humidified incubator with 5% CO_2 at 37°C. Gleevec was kindly provided by Novartis Pharma AG (Basel, Switzerland). Gleevec stock solution was made at a concentration of 30 mM in DMSO, and aliquots were kept at −20°C. The day after plating, cells were cultured in serum-free medium with 20 and 30 μM of Gleevec or DMSO vehicle, incubated for 48 h and then analysed for TUNEL and bromodeoxyuridine (BrdU).

Assessment of DNA synthesis by BrdU incorporation and apoptosis assay

For the proliferation (BrdU) and apoptosis (TdT mediated dUTP Nick End Labelling, TUNEL) analysis, cells were plated in six well dishes in 10% FBS at a cell density of 2×10^5 (HCT15, HCT116) and $2{,}6\times10^5$ (Co115). For proliferation analyses, cells were labelled by incubation in 10 μM of BrdU for 1 h, fixed with 4% paraformaldehyde, and nuclear incorporation was detected by immunofluoresce assay using an anti-BrdU antibody (Dako®). The proportion of positive nuclei (BrdU index) was determined from a count of >500 cells per datum point. For apoptosis analyses, cytospin preparations of both floating and attached cells were collected from each sample and fixed with 4% paraformaldehyde (15 min) at room temperature. Cells were washed in PBS and permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate (2 min) on ice. TUNEL analysis was performed using the "In situ cell death detection kit, fluorescein" from Roche®, following the manufacturer's instructions.

Results

Immunoexpression and mutational analysis of c-KIT in CRC

We found immunoreactivity in 11 of 73 (15%) primary CRCs. These 11 tumours displayed cytoplasmic and/or membrane c-KIT expression in 5–30% of the neoplastic cells (Fig. 1).

Thirty six lymph node metastases were also analysed for c-KIT expression, and 5 of 36 (14%) were positive (Fig. 2). In one metastasis, c-KIT expression was also found in the primary tumour. In the remaining four positive metastases,

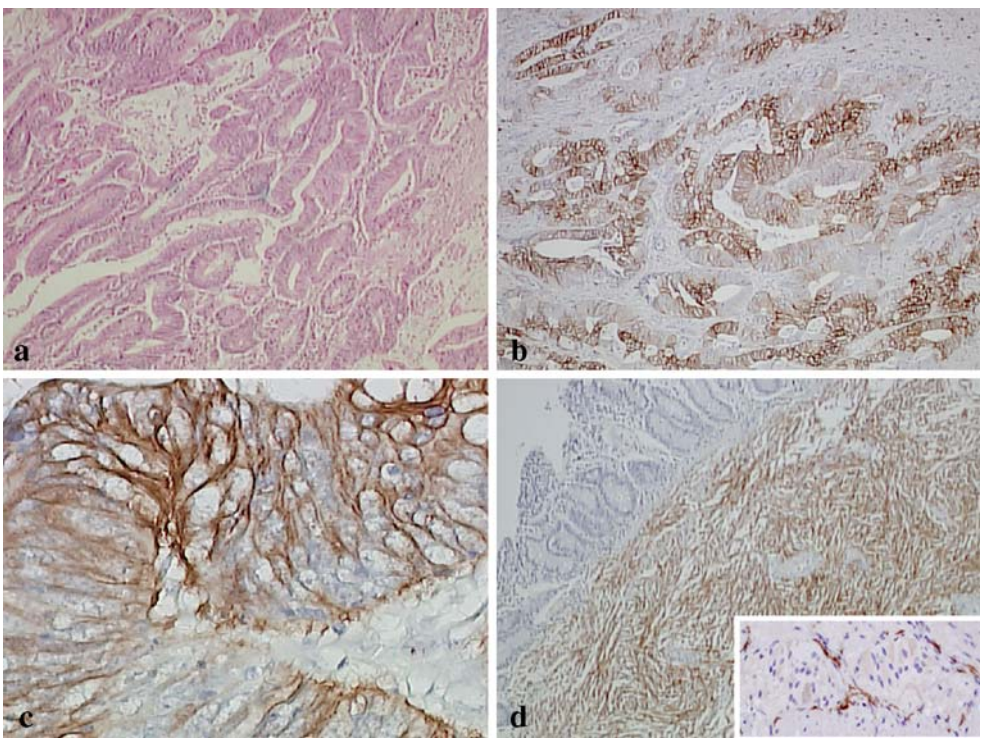

Fig. 1 Expression of c-KIT (CD117) in colorectal carcinomas (**a**, glandular features) showing cytoplasmic/membrane (**b**) and predominantly membrane (**c**) location. Note internal (positive mast cells in the upright stroma of **b**) and external positive controls [enteric GIST (**d**) and interstitial cells of Cajal (**d** inset)]. **a** H&E×5; **b**, **d** ABC×5 and **c** ABC×40

the corresponding primary tumours were negative for c-KIT immunoexpression.

We performed statistical analysis comparing c-KIT positive tumours with age, location, stage and Dukes classification, and no statistical significant correlation was found.

All primary tumours and metastases showing c-KIT immunoreactivity were screened for mutations in the *c-kit* gene. The mutation screening was restricted to the hotspot regions of the gene (exons 9, 10, 11, 13 and 17) by direct sequencing. None of the cases showing immunoreactivity for c-KIT displayed mutations in the hotspot regions of the *c-kit* gene (Fig. 2).

KRAS and *BRAF* mutational analysis, correlation with c-KIT expression

KRAS and *BRAF* genes were screened for mutations in all 73 primary CRCs analysed for c-KIT expression. We found *KRAS* mutations in 41/73 (56%) and *BRAF* mutations in 17/73 (23%) of the cases. Seven of eleven (64%) primary CRC displaying c-KIT expression had mutations at *KRAS* and/or *BRAF*, three CRC had mutations at *KRAS* only and four had mutations both in *KRAS* and *BRAF*. Four of the 11 cases with c-KIT expression did not show *KRAS* or *BRAF* genes.

KRAS and *BRAF* mutations were also screened in 36 lymph node metastases analysed for c-KIT expression, five of which with c-KIT imunoexpression. From these five c-KIT positive metastasis, three (60%) had mutations at *KRAS* and/or *BRAF*—two at *KRAS* only and one both at *KRAS* and *BRAF*.

Cellular effect of STI571/Gleevec in CRC-derived cell lines displaying different genetic profiles

To investigate the cellular effects of STI571/Gleevec in CRC, we selected three CRC-derived cell lines harbouring different genetic alterations: HCT116 harbours a $KRAS^{G13D}$ mutation and expresses c-KIT, CO115 harbours a $BRAF^{V600E}$ mutation and does not express c-KIT and HCT15 harbours a $KRAS^{G13D}$ mutation and does not express c-KIT ([22]; data not shown). We treated the three CRC cell lines with 20 and 30 μM of STI571/Gleevec for 48 h and studied cell proliferation by BrdU incorporation analysis and apoptosis by TUNEL analysis. Our results showed that STI571/Gleevec leads to a significant inhibition of BrdU incorporation in all the cell lines analysed ($p<0.05$). The inhibition was more effective using a dose of 30 μM (Fig. 3). Concerning apoptosis analyses, our results showed that STI571/Gleevec was able to induce apoptosis in all the cell lines, reaching statistical significance at a dose of 30 μM ($p<0.05$; Fig. 4).

Discussion

Several reports described the expression of c-KIT tyrosine kinase receptor in CRCs. While some authors argue that c-KIT expression is a rare event in CRC [26, 40], more recently, several authors detected c-KIT-positive staining in CRC ranging from 10 to 25% [6, 7, 28]. In the present study, we analysed a large series of primary CRC and

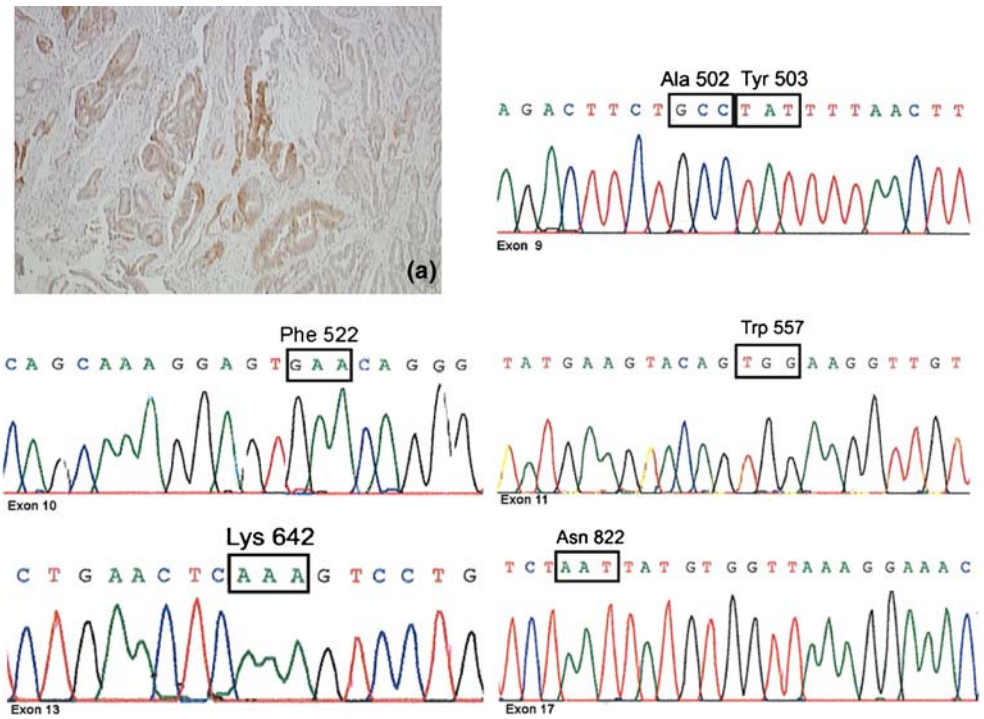

Fig. 2 Expression of c-KIT in a lymph node metastasis (**a**) and corresponding representative fragments of the sequencing analysis of *c-kit* exons 9, 10, 11, 13 and 17. Examples of commonly mutated codons in those exons are *highlighted*.
a ABC×5

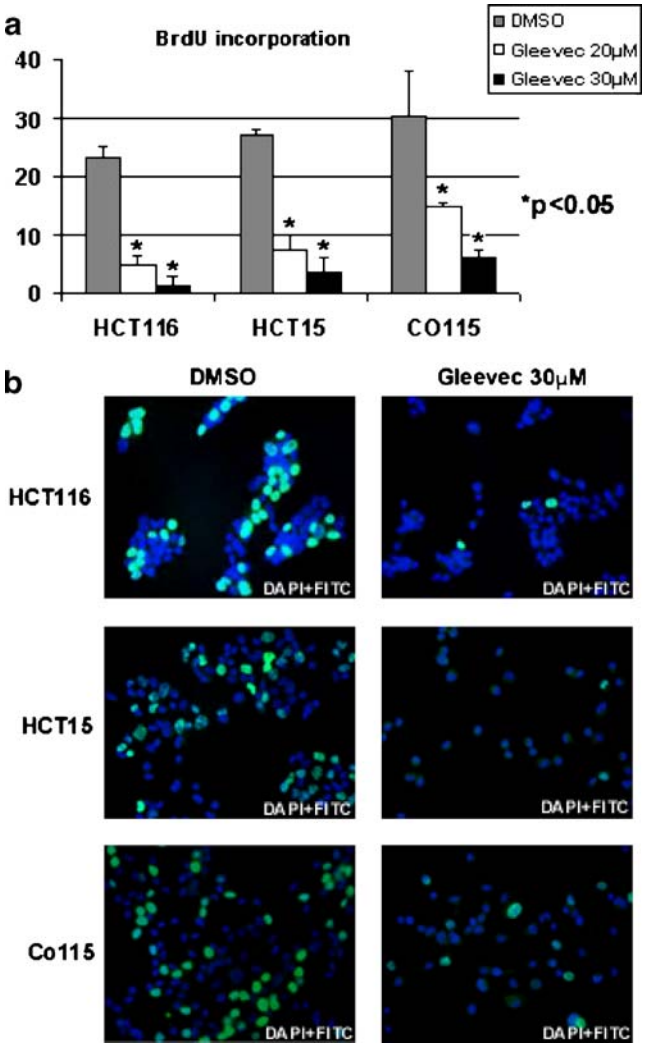

Fig. 3 **a** Representative proliferation graphs of bromodeoxy uridine (BrdU) analysis in HCT116, HCT15 and CO115 colorectal carcinoma-derived cell lines. Cells were treated with the vehicle control (DMSO) and different doses of Gleevec (20 and 30 μM) for 48 h. Significative inhibition of proliferation was observed in all the cell lines analysed with both doses ($p<0.05$, Gleevec 20 μM: $p=0.002$ for HCT116, $p=0.003$ for HCT15 and $p=0.007$ for CO115; Gleevec 30 μM: $p<0.0001$ for HCT116, $p=0.003$ for HCT15 and $p=0.003$ for CO115). **b** Representative photomicrographs of BrdU incorporation (positivity is shown by FITC-stained cells). Nuclei of the cells are counterstained with DAPI (magnification ×40)

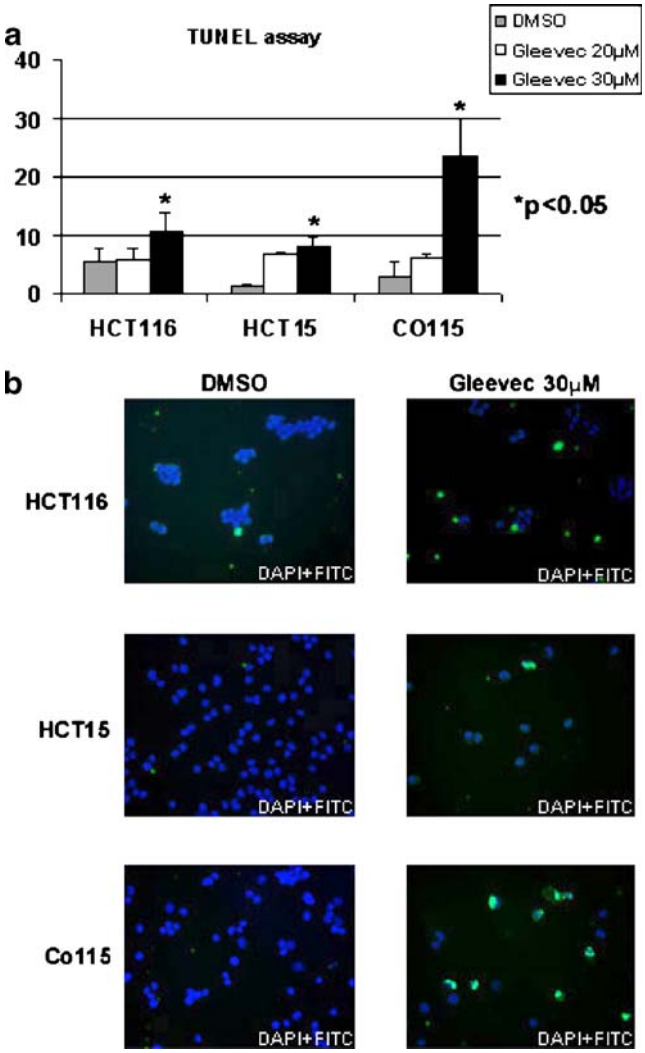

Fig. 4 **a** Apoptosis analysis by TUNEL assay in HCT116, HCT15 and CO115 colorectal carcinoma-derived cell lines representative graphs. Gleevec induced significative apoptosis in all the cell lines analysed at a dose of 30 μM after 48 h of treatment ($p<0.05$, Gleevec 30 μM: $p=0.03$ for HCT116, $p=0.02$ for HCT15 and $p=0.006$ for CO115). **b** Representative photomicrographs of TUNEL analysis in HCT116, HCT15 and CO115 treated with DMSO or Gleevec 30 μM. DAPI: counterstained to detect nuclei in FITC-stained cells in TUNEL assay (magnification ×40)

respective metastases and found c-KIT immunoreactivity in 15% of primary CRC and in 14% of CRC metastases. Our results are similar to the ones published in the latest reports. This similarity is reinforced by the fact that the same antibody, which is recommended for GIST diagnosis, was used [6, 7, 28]. To the best of our knowledge, this is the first report describing c-KIT expression in CRC metastases.

The biological role of c-KIT activation was investigated in CRC cell lines, and it was shown that it stimulates growth [6], protects colon carcinoma cells from apoptosis and enhances their migration and invasive potential [4]. Moreover, it was demonstrated that inhibition of c-KIT by STI571/Gleevec in c-KIT expressing CRC cell lines blocked cellular invasion [2, 5, 41]. Altogether, these results suggest a possible role for c-KIT in the invasive potential of the cells. In the present study, we observed that five metastases displayed c-KIT immunoexpression, four (80%) of which showed “de novo” expression of c-KIT protein that was not observed in the corresponding primary tumours. This observation suggests that c-KIT expression may have been acquired during metastization and is in accordance with its putative role in cell invasion.

Activating mutations in the *c-kit* gene were found associated with overexpression of c-KIT protein, namely, in GISTs [18, 21]. Nevertheless, it was already reported that in ovarian cancers, c-KIT expression by immunohistochemistry is not associated with mutations in the *c-kit* gene [35]. None of the previous reports on c-KIT expression in CRC performed mutation analysis of the *c-kit* proto-oncogene. We screened specifically *c-kit* exons previously appointed to be *hotspots* for mutations in tumour models as melanoma, intracranial germinomas and GISTS [8, 21, 27, 31, 37, 38]. To the best of our knowledge, somatic mutations in *c-kit* were never described outside of these *hotspot* exons in any cancer model, even in studies where all exons were screened.

Herein, we showed that c-KIT immunopositive CRC cases do not harbour mutations in the hotspot regions of *c-kit* gene. The mechanism by which c-KIT is overexpressed in CRCs remains to be clarified. Recently, *c-kit* amplification was described in gliomas as one of the underlying mechanisms leading to c-KIT overexpression [24]. Moreover, Bellone et al. [7] showed that the c-KIT ligand, SCF, was co-expressed in the majority of c-KIT-positive CRC, suggesting the possibility of c-KIT expression by an autocrine stimulation mechanism. In the present study, we did not analyse *c-kit* amplification nor SCF expression; thus, we cannot rule out these mechanisms as promoters of c-KIT overexpression in our CRC cases.

Others and our group described the presence of *KRAS* and *BRAF* mutations in a subset of CRC [9, 10, 22, 23]. Sixty four percent of the cases with c-KIT expression had *KRAS* or *BRAF* mutations. Our results suggest that c-KIT positive expression may co-exist with other oncogenic activation events, namely, *BRAF* and *KRAS*, in contrast to what was described for melanomas in which *BRAF* kinase-activating mutations are alternative to *c-kit* activating mutations [37, 38]. We would expect that in some cases of CRC, *c-kit* gene alterations could play a role in alternative to *BRAF* and *KRAS* as it was described that c-KIT pathway also signals to MAPK pathway. However, the results obtained did not support our initial hypothesis. In CRC, c-KIT expression seems to be independent of the activation of both *BRAF* and *KRAS* oncogenes.

STI571/Gleevec is a tyrosine kinase inhibitor. Several studies have already shown that STI571/Gleevec is able to inhibit proliferation, induce apoptosis and inhibit invasion in CRC cell lines [2, 5, 41]. In our study, we aimed to study the effect of STI571/Gleevec in cell lines with different genetic profiles. Our results showed that STI571/Gleevec was able to inhibit proliferation and induce apoptosis in all cell lines analysed independently of expressing c-KIT or harbouring mutations at *KRAS* or *BRAF* genes. As it has been shown that STI571/Gleevec inhibit other tyrosine kinase receptors as PDGFR [3, 17], we hypothesise that in two cell lines lacking c-KIT expression, PDGFR overexpression could be a possible mechanism by which STI571/Gleevec would act on those cell lines. However, the analysis of this gene by reverse transcriptase-PCR showed no PDGFR expression in both cell lines lacking c-KIT (data not shown). Accordingly, we hypothesise that STI571/Gleevec might interact with other signalling pathways involved in CRC cell survival. The mechanism by which STI571/Gleevec induces apoptosis in these cell lines needs to be further clarified. Moreover, our results show that c-KIT expression is not a predictor of STI571/Gleevec sensitivity in CRCs.

In conclusion, our results show that a subset of primary colorectal tumours and metastases express c-KIT in the absence of *c-kit*-activating mutations in *hotspot* regions, and this expression is not alternative to *BRAF* and/or *KRAS* activation. Moreover, our results suggest that STI571/Gleevec may be considered as another potential drug for CRC treatment because of its capacity to inhibit cell proliferation and promote apoptosis of tumour cells, regardless of the genetic background and by a still unknown mechanism.

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