Promoting role of cholecystokinin 2 receptor (CCK2R) in gastrointestinal stromal tumours pathogenesis

Short running title: Role of gastrin receptor in GIST development

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Abstract

The cholecystinin 2 receptor (CCK2R/CCKBR) is expressed in gastrointestinal stromal tumours (GIST). We sought to investigate the role of CCK2R in GIST pathogenesis. Molecular characterization of CCK2R was performed on a heterogeneous cohort of 50 GIST. In addition, CCK2R expression was evaluated by immunohistochemistry (IHC) using tissue microarray (TMA) containing 292 GIST, two cases of hyperplasia of interstitial Cajal's cells (ICC) and six gastric microscopic GIST.

Mono-allelic loss of the *CCK2R*/11p15 allele was identified in 13.7% of GIST, having no impact on the level of *CCK2R* transcript expression. No *CCK2R* mutations were found. The *CCK2Ri4sv*, CCK2R splice variant with retention of intron 4, was detected in 6 out of 20 analysed tumours. Wild-type *CCK2R* transcripts were commonly expressed (57.1% of cases) and this expression highly correlated with gastric primary site of GIST ($p < 0.001$). On protein level, expression of CCK2R in incidental ICC hyperplasia and early stages of gastric GIST development was documented, and its gastric association was confirmed on GIST-TMA by IHC.

To explore the *in vivo* effect of CCK2R vation on tumour growth, gastrin *versus* placebo was administered intra-peritoneally in nude mice carrying human GIST xenografts. The tumour volume was followed up for ten weeks. The effect of this stimulation on tumour cell proliferation/apoptosis was assessed by IHC, and KIT/PKC-theta signalling was evaluated by Western blotting (WB). *In vivo* experiments showed a two-fold increase in the volume of tumours which were exposed to gastrin in comparison with non-exposed controls ($p = 0.03$), with a significant increase in mitotic activity ($p = 0.04$) and Ki-67 proliferation index ($p = 0.008$). By

WB, gastrin stimulation resulted in hyper-activation of KIT and PKC- θ kinases, and in evident PI3K/AKT pathway over-activation.

Our results indicate a promoting role of CCK2R on GIST tumourigenesis, particularly in tumours of gastric origin.

Key words: Gastrointestinal stromal tumours, gastrin, CCK2R, micro-GIST, PKC- θ , stomach.

Introduction

Gastrointestinal stromal tumours (GIST) are clinically and histologically heterogeneous neoplasms that exhibit interstitial cells of Cajal (ICC) differentiation [1, 2]. They occur predominantly in the stomach (50-60%) and small intestine (10-20%). In majority GIST are driven by oncogenic *KIT* or *PDGFRA* mutations, which are crucial therapeutic targets [3-5]. GIST have been estimated to occur with a frequency of 13.6/million inhabitants [6], but minimal incidental GIST (<1 cm in size; micro-GIST) are far more common (in 20-30% of elderly individuals) [7-9]. Based on this statistics, micro-GIST likely represent pre-neoplastic lesions that might require additional genetic events or promoting external stimuli for evolution [7-11]. Cytogenetic and molecular studies have revealed compelling correlations between the biologic progression of GIST and chromosomal/genomic alterations [12, 13]. Whether the specific microenvironment related to particular anatomic site of origin plays a role in GIST development remains unclear.

Of particular interest in relation to GIST is the fact that specific receptors, such as cholecystokinin 2/gastrin (CCK2R), bombesin subtype 2, or neurotensin 1 (NTSR1) are frequently expressed in GIST, emerging as a potential targets for cancer therapy [14-18].

The CCK2R, formerly named CCKB/gastrin receptor, belongs to the superfamily of the 7-transmembrane alpha-helical domain G-protein-coupled receptors. The CCK2R is widely expressed in the central nervous system, where cholecystokinin is its endogenous ligand. In periphery, it is located on parietal and enterochromaffin-like (ECL) cells within the gastric epithelium, where it mediates the action of gastrin [19, 20]. CCK2R has been shown to have growth-promoting effects on pre-neoplastic gastrointestinal cells when stimulated by gastrin [21-25]. Gastrin-dependent activation of the CCK2R may activate important effectors involved in proliferation and survival such as the protein kinase C (PKC) and protein kinase B (PKB/AKT), throughout the direct interaction [26-30]. Interestingly, the CCK2R has been described to be well expressed in many neoplasms of different origin (e.g. neuroendocrine tumours and adenocarcinomas), while not being present in the corresponding normal tissues, indicating an important role in carcinogenesis [19, 31].

Human gastrointestinal (GI) tract cancers express at least three variants of the *CCK2R*: (1) *CCK2R*, the original variant identified as the receptor mediating normal gastric acid secretion and ECL proliferation, (2) an N-terminal truncated form of *CCK2R* (Δ *CCK2R*), and (3) *CCK2Ri4sv*, which encodes isoform containing an additional 69 amino-acid residues in its third intracellular loop domain [32-34]. Importantly, *CCK2Ri4sv* is reported to exhibit agonist-independent activation of the proto-oncogene SRC in pancreatic cancer [35], and constitutive activation of PKB/AKT in an oesophageal cancer cell line [30]. Although the expression of CCK2R in GIST is well recognized, the incidence and functional significance of these receptors

in GIST were not investigated. Given the potential clinical importance, we performed molecular characterization of CCK2R in a broad collection of heterogeneous type of GIST and explored the hypothesis that CCK2R receptor contribute to GIST growth.

Materials and methods

The study was approved by the Ethical Committee of KU Leuven (ML7481). Frozen samples from 50 resected imatinib-naïve GIST, originating from 49 patients (17 female and 32 male; age range 33-83 years), were selected from the files of Department of Pathology, KU Leuven. These were heterogeneous tumours with regard to primary anatomical site, *KIT/PDGFRA* genotype, and histopathological grade (35 primary and 15 malignant GIST). Tumour risk assessment was performed using AFIP criteria [1] (Table 1).

In addition, two cases of ICC hyperplasia, found in colon and small intestine in male patients with diverticular disease, were studied, as well as six cases of gastric micro-GIST coincidentally found in patients (four male and two female; age range 55-76 years) with gastric carcinoma.

Histopathological examination was performed on formalin fixed, paraffin embedded (FFPE) tissue. Four µm sections were used for routine haematoxylin and eosin (H&E) staining.

Immunohistochemistry (IHC) was performed using antibodies against CD117 (dilution 1:250; DAKO), CD34 (dilution 1:10; Becton Dickinson), CCK2R (dilution 1:300; EB06767, Everest Biotech) and gastrin (dilution 1:200; Thermo Scientific). The Envision⁺ System-HRP labelled polymer (DAKO) was used for the development of the staining.

Fluorescence in situ hybridization (FISH)

Genomic copy number changes were investigated by FISH on FFPE tumour sections, by co-hybridization of SpectrumOrange-labelled, bacterial artificial chromosome DNA RP11-98L18 (specific for *CCK2R*/11p15.4) and SpectrumGreen-labelled chromosome 11 centromeric (CEP11; Abbott Laboratories) probes. Probe labelling, hybridization, and criteria for evaluation were carried out as described previously [36].

Mutational analysis

DNA was isolated, and *KIT*/*PDGFRA* mutations were assessed as reported [37].

The *CCK2R* exons 1-5 were amplified by polymerase chain reaction (PCR) using standard Taq DNA polymerase (Roche) and sequenced bi-directionally using ABI PRISM 2700 (Applied Biosystems). The specific primers for amplified fragments were designed using Primer3 software (Supplementary Table S1).

Quantitative Reverse Transcriptase PCR (qRT-PCR)

RNA was isolated from frozen tissues with the RNeasy Mini Kit (Qiagen), including a DNase I digestion step. Reverse transcription was performed with SuperScript III (Invitrogen).

For *qRT-PCR* two different reactions were carried out to amplify either total *CCK2R* or *CCK2Ri4sv* cDNA. The cDNA corresponding to 100 ng total RNA was amplified in a 15 µl reaction volume using qPCR MasterMixPlus for SYBR®Green I w/o UNG (Eurogentec) in Light Cycler 480 (Roche). For normalization, endogenous reference genes *GUSB* and *HPRT* were used. As positive and negative controls, the previously described [38, 39] human oesophageal squamous carcinoma cell lines stably expressing or not-expressing wild-type *CCK2R* [OE33GR(+) vs. OE33 cells], the kind gift from Dr. Jankowski (University of Liverpool, UK), were used. Two sporadic leiomyosarcomas and normal brain, stomach and liver were also studied. Primers for analysed genes were designed using Light Cycler Probe Design2 (Roche) (Supplementary Table S1).

For statistical analysis, the Mann Whitney U-test was performed, using the software package STATISTICA (StatSoft Inc, USA).

End-point PCR

In 20 GIST positive for *CCK2R* expression, possible *CCK2R* transcript variants were investigated by a nested PCR, in which the first round reaction amplified total *CCK2R* cDNA, and the second round reaction either wild-type (WT) *CCK2R* or *CCK2Ri4sv*, as described [32]. Normal brain and liver tissues were also investigated. Amplicons were sequenced to confirm their identities using bi-directional Sanger sequencing. The end-point PCR protocol is presented in Supporting materials. The primers design is shown on Fig. 2B and their sequences are presented in Supplementary Table S1.

CCK2R/gastrin expression by IHC

The expression of gastrin and CCK2R on protein level was investigated by IHC using GIST-TMA containing a heterogeneous set of 292 FFPE tumours, micro-GIST and on cases of ICC hyperplasia (detailed in Supplementary Table S2). Normal stomach specimens were included in the study as positive control. The protocol for CCK2R/gastrin immunostaining is presented in Supporting materials. Staining was regarded positive when specific (i.e. above background) CCK2R/gastrin immunostaining signal was present in >10% of tumour cells.

For statistical analysis, TMA data were fitted using a multivariate logistic regression model with the binary variable CCK2R protein expression (0/1 or negative/positive) as a response variable. The patient gender, tumour site, histology grade and *KIT/PDGFR*A genotype were used as explanatory variables. The analysis was performed using SAS software, version 9.2 of the SAS System for Windows.

In vivo study

Nude mice (adult female NMRI^{nu/nu}, n=8) were grafted bilaterally with recurrent gastric GIST (carrier of *KIT*-V560D mutation), in which *CCK2R* expression was confirmed by quantitative and nested RT-PCR. Animals were randomized into two groups and injected intra-peritoneally either with vehicle only (0.9% NaCl; group 1) or with CCK2R agonist, gastrin (Sigma-Aldrich), at 10 ng/kg/every 3rd day (group 2). Tumour volume was assessed with Vernier's calliper every second day, as described [40]. After 10 weeks of treatment mice were sacrificed and xenografts tissues were partially preserved in formalin or snap-frozen for further evaluation.

Tumour proliferation indexes were assessed by routine H&E and Ki-67 staining as described [40]. In short, mitoses were counted in 10 high power fields (HPF) at 400-fold magnification on

H&E slides. For Ki-67 proliferative index, IHC was performed using anti-Ki-67 monoclonal rabbit clone SP6 antibody (1:200, Thermo Scientific). Ki-67 index was determined by evaluating the average percentage of positive nuclei in three HPF. Statistics were performed using Mann-Whitney U test (STATISTICA software v 9.0).

The KIT/ PKC-theta signalling was evaluated by Western blotting (WB) as reported [37] , using antibodies against total KIT (DAKO), total MAPK (Zymed Laboratories), total PKC-theta (Santa Cruz Biotechnology), phospho-KIT, phospho-PKC-theta, phospho-AKT, total AKT, phospho-MAPK, phospho-S6, total S6 and cleaved caspase-3 (Cell Signaling). An antibody against B-actin (Sigma-Aldrich) was applied as a protein-loading control. The HRP-conjugated anti-rabbit or anti-mouse IgG (DAKO) was used as a secondary reagent and proteins were visualized with Enhanced Chemiluminescence (Thermo Scientific), which was captured with the FUJI mini-LAS4000 imaging system. Densitometry analysis was performed using AIDA software (Raytest). The optical density of each band was measured and normalized against B-actin.

Results

Histopathology

On histology, the tumours showed a spindle, epithelioid or mixed histotype, typical of GIST. By IHC, the vast majority (98%) were KIT/CD117 immunopositive. CD34 positivity was found in 62.7% of tumours and predominantly in GIST of gastric origin ($p=0.001$; chi-square test) (Table 1).

Two cases of sporadic ICC hyperplasia presented a focal proliferation of CD117/CD34 immunopositive cells (3-5 mm of size) within the inner layer of the *muscularis propria*. In both cases mutation analysis revealed wild-type *KIT/PDGFR*A genotype.

All micro-GIST (size 3-15 mm; mean 9 mm) originated from gastric *atrium* or *fundus*. Histology revealed uniform spindle cell morphology with low (0-3/50 HPF) mitotic activity. All were KIT/CD34 immunopositive. Two cases revealed mutations in *KIT* exon 11 (p.W557_K558del and p.W557R, respectively).

Molecular characterization and clinico-pathological correlations of CCK2R expression in GIST

By FISH, we investigated the genomic copy number changes of the *CCK2R* gene, which maps to chromosome 11p15.4; this region is frequently lost during malignant progression of GIST (Fig. 1). Mono-allelic loss of the *CCK2R*/11p15 allele was identified in 13.7% of the specimens (Table 1). Notably, this loss did not have significant impact on the level of *CCK2R* transcripts evaluated by qRT-PCR (p=0.2).

By direct sequencing, no mutations were identified in *CCK2R* in any of the examined cases.

With regard to *CCK2Ri4sv* transcripts, expression levels were close to the detection limit of qRT-PCR. However, the end-point PCR analysis revealed the presence of *CCK2Ri4sv* in six out of 20 analysed GIST (Fig. 2A). Of note, all *CCK2Ri4sv* positive tumours were of gastric origin and of low malignancy potential. Splice variant mRNA was not identified in the normal tissues. The presence of *CCK2Ri4sv* transcript was confirmed by sequencing.

By qRT-PCR, *CCK2R* transcripts were commonly expressed from low-risk to overtly malignant GIST (57.1% of tumours). This expression highly correlated with gastric primary site (gastric 75% vs. non-gastric 32%; $p<0.001$), irrespective of the *KIT/PDGFRA* genotype. In addition, the level of *CCK2R* transcript expression was eight folds higher in tumours of gastric when compared to GIST of non-gastric origin ($p=0.001$). The low/intermediate-risk GIST expressed *CCK2R* more frequently than high-risk/overtly malignant tumours (74.8% vs. 41.4% respectively; $p=0.01$) (Table 1). Stomach and whole brain were positive for the total *CCK2R* transcripts, whereas liver and the leiomyosarcomas were negative.

CCK2R expression was also explored on the previously published normalized gene expression data of a cohort of 62 primary GIST [13], using t-test. This analysis confirmed that *CCK2R* is under-expressed in intestinal vs. gastric GIST (log fold change intestinal vs. gastric = -3.2) and lower in high- vs. low/intermediate-risk tumours (log fold change low/intermediate vs. high risk = 2.8).

IHC analysis

The results of *CCK2R* expression frequency and clinico-pathological correlations on protein level were validated and confirmed by IHC using GIST-TMA. *CCK2R* immunopositivity was disclosed in 60.9% of the specimens, with either a cytoplasmic or mixed (i.e. cytoplasmic and nuclear) immunostaining pattern (Fig. 3C and D). The nuclear localization is believed to be due to a rapid internalization and translocation of the receptor to the nucleus, bearing still unknown functional significance [41]. Negative *CCK2R* immunostaining was found in adjacent non-tumoral tissues and controls (Supplementary Fig. S1). By univariate analysis, gastric primary location, presence of *KIT* exon 11 mutation and low/intermediate malignancy risk correlated significantly with *CCK2R* immunopositivity. However, in multivariate analysis only gastric site

of origin remained highly statistically significant [$p < 0.0001$; OR 4.6 (2.3-9.1), 95% CI] (Supplementary Table S2).

By IHC, both cases of focal ICC hyperplasia showed CCK2R immunopositivity (Fig. 3E and F). Also all gastric micro-GIST showed clear CCK2R protein expression, albeit with varying level of staining intensity (Fig. 3G and H).

Negative gastrin immunostaining was observed in all cases on GIST-TMA. Similarly, negative results were obtained in cases of ICC hyperplasia and micro-GIST, though positive gastrin immunostaining was detected in G cells of gastric mucosa specimens that were used as positive control (Supplementary Fig. S2).

In vivo effect of CCK2R activation by gastrin on tumour growth, cell proliferation and KIT signalling

To assess the effects of CCK2R activation *in vivo*, mice carrying GIST xenografts were stimulated with gastrin for ten weeks. Remarkably, a two-fold volume increase of tumours exposed to gastrin in comparison with non-stimulated controls ($p = 0.03$) was observed (Fig. 4A and B). In addition, the proliferative activity was significantly increased under gastrin stimulation, as evidenced by higher mitotic count and Ki-67 labelling index in tumours from gastrin-exposed vs. control mice ($p = 0.04$ and $p = 0.008$, respectively) (Fig. 4C and D).

By WB, gastrin stimulation resulted in increased phosphorylation of KIT kinase (p-KIT^{Y703} and p-KIT^{Y719} increased by 1.4- and 1.3-folds, respectively), and in a remarkable increase of PKC- θ kinase activation (22-folds), resulting in evident AKT (2.5-folds), MAPK (8.0-folds), and S6 (1.3-folds) hyperactivation. The apoptotic index was not affected (Fig. 5).

Discussion

In this study, characterization of CCK2R in GIST was performed in search for potential clinico-pathological correlations. Analysis on genomic level revealed mono-allelic loss of *CCK2R* in 13.7% of cases, but this loss did not have impact on receptor expression. No somatic *CCK2R* mutations were found. The potential role of *CCK2Ri4sv* in GIST pathogenesis was suggested by Korner and co-workers [32], who found *CCK2Ri4sv* transcripts in all (n=4) examined samples. In our study, the presence of the *CCK2Ri4sv* in GIST was confirmed, albeit with lower frequency (6 out of 20 analysed GIST). *CCK2Ri4sv* has previously been shown to stimulate cell proliferation *in vitro* and *in vivo* [33, 35]. Nevertheless, we found the *CCK2Ri4sv* to be present only in low/intermediate risk tumours, with 4 out of 6 positive cases presenting low ($\leq 5/50$ HPF) mitotic index. Thus, the *CCK2Ri4sv* seems to have a marginal role in GIST progression, similarly to what is observed in other tumour types.

We confirmed by qRT-PCR that *CCK2R* is frequently expressed in GIST, as indicated before [14-16]. Notably, the incidence and the level of *CCK2R* mRNA expression were highly correlated with gastric site of tumour origin. Moreover, the level of expression was higher in tumours of low malignancy potential in comparison with high-risk/malignant ones, suggesting a possible role of CCK2R in early stages of GIST development. These associations were confirmed by an *in silico* analysis of a previously published gene expression data set of a cohort of 62 primary GIST [13]. Our results were corroborated on protein level by IHC performed on GIST-TMA. The difference in frequency of CCK2R expression between tumours of gastric and non-gastric origin was highly statistically significant ($p < 0.0001$). This clearly illustrates that CCK2R expression predominantly depends on tumour primary site.

Importantly, we found that CCK2R was also expressed in incidental ICC hyperplasia and gastric micro-GIST. Microscopic foci of sporadic KIT-positive spindle cell ICC hyperplasia and better-delineated micro-GIST (tumours <1 cm; designated also as “GIST tumourlets” or “seedling GIST”) have been introduced as terminology for small preclinical lesions that most likely need additional stimuli to evolve into clinically relevant GIST [7]. Incidental micro-GIST are common in gastro-oesophageal resections (2.9-35%), contrasting with their intestinal counterparts ($\leq$ 0.1%) [7-11]. In a recent study of 170 GIST of small size, the vast majority (77%) of micro-GIST were located in the stomach [42]. The remarkable variation in the incidence of micro-GIST at different GI tract sites suggests an origin from heterogeneous subsets of ICC with varying potentials for neoplastic transformation. Alternatively, other factors might contribute to GIST growth and progression, such as gastric-site dependent growth factors and/or mitogens released by the gastric mucosa cells or other components of motility-related mechanisms in the stomach microenvironment. Given the common expression of CCK2R in pre-clinical lesions (ICC hyperplasia and micro-GIST) and clinical GIST from the stomach, it is tempting to suggest a possible role of gastrin and CCK2R in early stages of GIST evolution, and particularly in tumours of gastric origin. In humans, clinical data from hypergastrinemic patients (such as those with gastrinoma or chronic proton pump usage) clearly demonstrate that hypergastrinemia alone does not increase the risk of gastric carcinoma [22, 43, 44]. Conversely, gastrin is up-regulated locally in 78% of premalignant adenomatous colon polyps, before the appearance of invasive carcinoma [44], and gastrin expression has been linked to key mutations in the initiation of colorectal carcinogenesis. In the development of colorectal tumours autocrine gastrin may also have a role, as expression occurs early in the adenoma-carcinoma sequence [22]. Our data indicate that GIST do not produce their own gastrin. Yet, its local production by G cells in the deep crypts of the stomach mucosa might give rise to the gastrin levels across the whole gastric

wall. In this context, it would be interesting to investigate whether systemic hypergastrinaemia leads to increased GIST incidence in a well-designed epidemiological study.

The novel findings coming from our *in vivo* experiments support the important role of gastrin and CCK2R in GIST pathogenesis. GIST xenografts stimulated by gastrin presented significant increase of tumour volume and tumour cell proliferation rates in comparison with the non-stimulated control. Moreover, remarkable increase in PKC-theta and KIT phosphorylation with the subsequent over-activation of major downstream KIT signalling pathways (AKT and MAPK) was observed. These data confirm the role of CCK2R in GIST pathogenesis, possibly through PKC-theta activation.

In summary, we have shown the expression of CCK2R in incidental ICC hyperplasia and early stages of gastric GIST development, associated with predominant CCK2R presence in tumours of gastric origin. *In vivo* study proved the promoting role of gastrin stimulation on GIST growth. Whether the inhibition of CCK2R can play a role in management of patients with GIST warrants further studies.

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Authors Contributions

AQ: execution of experiments, generation and analysis of data, BD: data generation and analysis; AW: informatics and data analysis; MB, GF, VV: data collection and interpretation; PS: data collection and additional infrastructural support; FC: pre-existing data analysis; J-MC: collection of tumours and TMA construction; RS: generation and interpretation of data, MDR: project conception, design and guidance of implementation. All authors were involved in writing the paper and approved the submitted versions.

List of online Supporting Information

End-point PCR protocol

GIST-TMA construction

CCK2R/gastrin IHC protocol

Supplementary_tables

Table S1. List of primers

Table S2. Clinico-pathological characteristics of patients and tumours used for GIST-TMA analysis

Supplementary_figures

Figure S1. Validation of anti-CCK2R antibody by absorption experiment.

Figure S2. Validation of anti-gastrin antibody.

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Figures

Figure 1. Dual-colour interphase FISH analysis on GIST specimen by co-hybridization of SpectrumOrange-labelled *CCK2R*/11p15 (RP11-98L18) DNA (red signals) and SpectrumGreen-labelled chromosome 11 CEP (green signals) probes. Mono-allelic *CCK2R* loss is evidenced by a single red hybridization signal in reference to two green signals in each of the tumour nuclei.

Figure 2. Nested PCR. A) Agarose gels showing representative end-point RT-PCR products: a) second round nested PCR products for *CCK2R* WT; b) second round nested PCR products for *CCK2Ri4sv*; c) *B-actin* control PCR, run for each samples. All GIST samples analysed expressed *CCK2R*, included GIST xenograft used for the *in vivo* study. *CCK2R* transcripts were present in normal brain tissue but not in normal liver. *CCK2Ri4sv* transcripts were detected only in 6 GIST cases. For negative control no template was added. 1 kb DNA ladder was used as molecular weight marker. Additional bands migrating with higher weights correspond to amplified genomic *CCK2R* or are non-specific as confirmed by sequencing. B) Primer design for nested PCR.

Figure 3. Representative examples of histological and immunohistochemical images on FFPE GIST and control tissues. A) Normal stomach (control): parietal cells within gastric mucosa stain positive for *CCK2R* (x20). B) *CCK2R* immuno-negative GIST (x200). C) Gastric GIST showing cytoplasmatic pattern of *CCK2R* immunostaining. Adjacent non-tumoral tissue (*) exhibited only minimal background signal (x200); insert: x400. D) *CCK2R* immunopositive GIST - cytoplasmic and nuclear (mixed) pattern of staining (x200). E) Incidental ICC hyperplasia in the colon, H&E (x20). Insert lower: H&E (x400). Insert upper: KIT immunopositive hyperplastic ICC cells (x400). F) *CCK2R* immunostaining of the incidental ICC hyperplasia in the colon

(x20). Insert: immunopositive for CCK2R hyperplastic ICC and ganglion cells of Auerbach's plexus (x400). G and H) Immunopositive for CCK2R micro-GIST (x100).

Figure 4. Effect of gastrin exposure on GIST xenografts. A) Tumour volume assessment: two-fold increase in the volume of gastrin stimulated tumours (gastrin) vs. controls ($p=0.03$). B) Tumour weight measurement showed more than 10% increment due to gastrin stimulation. C) A statistically significant increase of Ki-67 proliferation index was observed for gastrin stimulated tumours ($p=0.04$). D) Mitotic activity was significantly higher in gastrin stimulated vs. not-stimulated tumours ($p=0.008$). Results are presented as box plots showing maximum, minimum, interquartile range and median.

Figure 5. Western Blot and densitometry analysis. A) Immunoblots showing the effect of gastrin stimulation on KIT/ PKC- θ signalling in GIST xenografts. Actin served as a loading control. B) Results of densitometry are presented according to the formula: $(\text{Average densitometry assessment gastrin stimulated tumours} / \text{Average densitometry assessment control tumours}) - 1$.

Table 1. The clinico-pathological and molecular data of 50 GIST retrieved for the study

No.	Age [years]	Sex	Primary site	Histotype	Size [mm]	MI [per 50 HPF]	IHC		Risk of primary*/ overtly malignant	<i>KIT/PDGFR</i> A exon mutation	<i>CCK2R</i> loss by FISH	CCK2R expression		
							CD117	CD34				RT- PCR	<i>CCK2Ri4sv**</i>	IHC
1	75	M	stomach	epith.	30	3	pos	pos	v. low	<i>PDGFRA</i> 14	no	pos	neg	pos
2	66	F	stomach	Mixed	35	4	pos	pos	v. low	<i>PDGFRA</i> 18	no	neg	nd	nd
3	77	F	stomach	epith.	60	4	pos	pos	low	<i>PDGFRA</i> 12	no	neg	nd	nd
4	55	M	stomach	Mixed	50	5	pos	pos	low	<i>PDGFRA</i> 14	no	neg	nd	nd
5	71	M	stomach	epith.	55	1	pos	pos	low	<i>PDGFRA</i> 18	no	pos	neg	nd
6	49	M	stomach	epith.	75	4	neg	pos	low	<i>PDGFRA</i> 18	no	neg	nd	nd
7	69	M	stomach	Spindle	35	4	pos	pos	low	<i>PDGFRA</i> 18	no	pos	neg	nd
8	76	F	stomach	epith.	30	17	pos	pos	intermed.	<i>PDGFRA</i> 18	no	pos	pos	pos
9	53	M	stomach	Mixed	55	4	pos	pos	intermed.	<i>PDGFRA</i> 18	no	pos	pos	pos
10	51	M	stomach	Spindle	180	2	pos	pos	high	<i>PDGFRA</i> 18	no	pos	nd	pos
11	61	M	stomach	Spindle	55	5	pos	pos	low	<i>KIT</i> 11	no	pos	pos	pos
12	53	M	stomach	epith.	6	5	pos	neg	low	<i>KIT</i> 11	no	pos	nd	pos
13	64	M	stomach	Spindle	45	9	pos	pos	low	<i>KIT</i> 11	no	pos	nd	pos
14	83	M	stomach	Spindle	50	4	pos	pos	low	<i>KIT</i> 11	no	pos	pos	nd
15	80	F	stomach	Spindle	55	3	pos	pos	low	<i>KIT</i> 11	no	pos	nd	nd
16	36	F	stomach	Spindle	35	60	pos	pos	intermed.	<i>KIT</i> 11	no	pos	neg	neg
17	67	F	stomach	Spindle	40	25	pos	pos	intermed.	<i>KIT</i> 11	no	pos	pos	nd
18	83	F	stomach	Spindle	85	7	pos	pos	intermed.	<i>KIT</i> 11	no	pos	pos	nd
19	56	M	stomach	Spindle	45	15	pos	pos	intermed.	<i>KIT</i> 11	no	pos	nd	nd
20	48	F	stomach	Spindle	50	37	pos	neg	high	<i>KIT</i> 11	no	pos	neg	pos
21	67	M	stomach	Mixed	NA	22	pos	pos	high	<i>KIT</i> 11	no	neg	nd	nd
22	49	M	stomach	Spindle	80	26	pos	pos	high	<i>KIT</i> 11	no	pos	nd	nd
23	57	M	stomach	Spindle	140	14	pos	neg	high	<i>KIT</i> 11	no	pos	neg	nd
24	43	M	stomach	Mixed	8	15	pos	neg	M (meta)	<i>KIT</i> 11	yes	neg	nd	nd
25	74	M	stomach	Spindle	110	30	pos	pos	M (rec)	<i>KIT</i> 11	no	pos	nd	pos
26	72	M	sm. int.	Spindle	48	4	pos	pos	low	<i>KIT</i> 11	no	pos	neg	pos

27	37	F	sm. int.	Spindle	120	1	pos	neg	high	<i>KIT</i> 11	no	pos	neg	nd
28	51	M	sm. int.	Spindle	120	15	pos	neg	high	<i>KIT</i> 11	yes	pos	nd	pos
29	78	M	sm. int.	Spindle	55	12	pos	neg	high	<i>KIT</i> 11	no	neg	nd	neg
30	51	M	sm. int.	Spindle	150	8	pos	neg	high	<i>KIT</i> 11	no	neg	nd	neg
31a	49	F	sm. int.	Spindle	90	8	pos	neg	high	<i>KIT</i> 11	no	neg	nd	neg
31b			sm. int.	Spindle	70	12	pos	neg	M (rec)	<i>KIT</i> 11	no	neg	nd	neg
32	78	M	sm. int.	Spindle	280	5	pos	pos	M (rec)	<i>KIT</i> 11	no	pos	neg	neg
33	59	M	sm. int.	epith.	NA	38	pos	pos	M (rec)	<i>KIT</i> 11	yes	neg	nd	nd
34	59	F	sm. int.	Spindle	100	52	pos	pos	M (meta)	<i>KIT</i> 11	no	pos	nd	pos
35	45	M	sm. int.	Spindle	90	40	pos	neg	M (meta)	<i>KIT</i> 11	no	neg	nd	nd
36	45	M	sm. int.	Spindle	30	20	pos	neg	M (rec)	<i>KIT</i> 11	no	neg	nd	neg
37	50	M	sm. int.	Spindle	70	10	pos	neg	M (meta)	<i>KIT</i> 11	yes	neg	nd	neg
38	68	F	duod.	Spindle	30	4	pos	pos	intermed.	<i>KIT</i> 11	no	pos	neg	nd
39	65	M	rectum	Spindle	55	26	pos	pos	M (meta)	<i>KIT</i> 11	no	neg	nd	nd
40	54	F	sm. int.	Spindle	65	13	pos	neg	high	<i>KIT</i> 9	no	neg	nd	nd
41	61	M	sm. int.	Mixed	38	7	pos	pos	high	<i>KIT</i> 9	no	neg	nd	nd
42	76	M	sm. int.	Spindle	85	55	pos	neg	M (meta)	<i>KIT</i> 9	yes	neg	nd	nd
43	44	M	perit.	Spindle	180	25	pos	pos	M (meta)	<i>KIT</i> 9	no	pos	neg	nd
44	50	F	sm. int.	Mixed	50	5	pos	neg	intermed.	<i>KIT</i> 9	no	neg	nd	neg
45	58	M	colon	Spindle	110	17	pos	neg	M (meta)	<i>KIT</i> 9	yes	neg	nd	neg
46	61	M	sm. int.	epith.	80	5	pos	pos	high	<i>KIT</i> 17	no	neg	nd	neg
47	50	F	stomach	epith.	45	18	pos	pos	M (rec)	WT	no	pos	nd	pos
48	56	F	stomach	Spindle	95	10	pos	neg	M (meta)	WT	no	neg	nd	neg
49	33	F	stomach	Mixed	multiple	46	pos	pos	high	WT	yes	pos	neg	pos

* According to AIFP classification; ** End-point analysis

Abbreviations: F-female; M-male; sm. int.-small intestine; duod.-duodenum; perit.-peritoneum; epith.-epithelioid; NA-not available; MI-mitotic index; HPF-high power field; IHC-immunohistochemistry; pos-positive; neg-negative; v. low-very low; interm.-intermediate; M-overtly malignant; meta-metastasis; rec-recurrent disease; WT-without detectable *KIT/PDGFRA* mutations; nd-not done

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5