Integrated genomic study of quadruple-WT GIST (KIT/PDGFRα/SDH/RAS pathway wild-type GIST)

Margherita Nannini¹, Annalisa Astolfi², Milena Urbini², Valentina Indio², Donatella Santini³, Michael C Heinrich⁴, Christopher L Corless⁵, Claudio Ceccarelli⁵, Maristella Saponara², Anna Mandrioli¹, Cristian Lolli¹, Giorgio Ercolani⁶, Giovanni Brandi¹, Guido Biasco¹,² and Maria A Pantaleo¹,²*

Abstract

Background: About 10-15% of adult gastrointestinal stromal tumors (GIST) and the vast majority of pediatric GIST do not harbour KIT or platelet-derived growth factor receptor alpha (PDGFRα) mutations (J Clin Oncol 22:3813–3825, 2004; Hematol Oncol Clin North Am 23:15–34, 2009). The molecular biology of these GIST, originally defined as KIT/PDGFRα wild-type (WT), is complex due to the existence of different subgroups with distinct molecular hallmarks, including defects in the succinate dehydrogenase (SDH) complex and mutations of neurofibromatosis type 1 (NF1), BRAF, or KRAS genes (RAS-pathway or RAS-P).

In this extremely heterogeneous landscape, the clinical profile and molecular abnormalities of the small subgroup of WT GIST suitably referred to as quadruple wild-type GIST (quadruple WT or KIT WT/PDGFRα WT/SDH WT/RAS-P WT) remains undefined. The aim of this study is to investigate the genomic profile of KIT WT/PDGFRα WT/SDH WT/RAS-P WT GIST, by using a massively parallel sequencing and microarray approach, and compare it with the genomic profile of other GIST subtypes.

Methods: We performed a whole genome analysis using a massively parallel sequencing approach on a total of 16 GIST cases (2 KIT WT/PDGFRα WT/SDH WT and SDHB IHC+ SDHA IHC+, 2 KIT WT/PDGFRα WT/SDH IHC mut and SDHB IHC+/SDHA IHC- and 12 cases of KIT IHC+ or PDGFRα IHC GIST). To confirm and extend the results, whole-genome gene expression analysis by microarray was performed on 9 out 16 patients analyzed by RNAseq and an additional 20 GIST patients (1 KIT WT/PDGFRα WT/SDHA IHC mut GIST and 19 KIT mut or PDGFRα mut GIST). The most impressive data were validated by quantitative PCR and Western Blot analysis.

Results: We found that both cases of quadruple WT GIST had a genomic profile profoundly different from both either KIT/PDGFRα mutated or SDHA-mutated GIST. In particular, the quadruple WT GIST tumors are characterized by the overexpression of molecular markers (CALCRL and COL22A1) and of specific oncogenes including tyrosine and cyclin-dependent kinases (NTRK2 and CDK6) and one member of the ETS-transcription factor family (ERG).

(Continued on next page)
Conclusion: We report for the first time an integrated genomic picture of KitWT/PDGFRAWT/SDH−/RAS−PDGFRA mut GIST, using massively parallel sequencing and gene expression analyses, and found that quadrupleWT GIST have an expression signature that is distinct from SDH-mutant GIST as well as GIST harbouring mutations in Kit or PDGFRA. Our findings suggest that quadrupleWT GIST represent another unique group within the family of gastrointestinal stromal tumors.

Keywords: Gastrointestinal stromal tumors (GIST), Wild-type, Kit, PDGFRA, Succinate dehydrogenase, SDHA, RAS, QuadrupleWT

Background

About 10-15% of adult gastrointestinal stromal tumors (GIST) and the vast majority of pediatric GIST do not harbour Kit or platelet-derived growth factor receptor alpha (PDGFRA) mutations [1,2]. These GIST were originally defined as Kit/PDGFRA wild-type (KitWT/PDGFRAWT) and generally are less sensitive to tyrosine-kinase inhibitors [3-5]. Their molecular biology is heterogeneous as evidence by the existence of different subgroups with distinct molecular abnormalities (Figure 1). KitWT/PDGFRAWT GIST can be divided into two main groups according to the succinate dehydrogenase subunit B (SDHB) immunochemical status (IHC): SDHB positive (SDHB+/IHC), or type 1 GIST which includes neurofibromatosis type 1 (NF1)-mutant GIST and some sporadic KitWT/PDGFRAWT GIST. The second group of KitWT/PDGFRAWT, called as type 2 GIST, is characterized by a lack of SDHB protein expression (SDHB−/IHC). In some cases SDHB−/IHC is due to germline and/or de novo mutations of any of the four SDH subunits (SDHA*mut) [6-8]. The SDHB−/IHC includes additional subgroups that can be distinguished on the basis of the SDHA IHC status, which strictly correlates with the presence of SDHA-inactivating mutations (SDHA*mut) [9-16]. In particular, SDHA+/IHC-SDHA+/IHC+ GIST include a subgroup of young adult women patients with a well defined clinical and biological profile, generally characterized by the gastric primary tumor localization, a predominantly mixed epithelioid and spindle cell morphology, diffuse IHC positivity for Kit and discovered on gastrointestinal stromal tumours 1 (DOG1), frequent lymph node metastases, and an indolent course of disease even if metastasis is present [17]. Moreover, they are characterized by overexpression of the insulin growth factor 1 receptor (IGFIR) [18-21]. On the contrary, SDHB−/IHC−, but SDHA−/IHC+ subgroup include 1) cases of syndromic GIST arising from the Carney-Stratakis Syndrome (CSS), that are characterized by SDHB, SDHC or SDHD inactivating mutations (SDHB*mut, SDHC*mut or SDHD*mut); and 2) cases of Carney Triad (CT), that lack SDHX-mutations [6,22-24]. More rarely, SDHB−/IHC−/SDHA−/IHC− sub-group may include sporadic KitWT/PDGFRAWT GIST characterized by SDHB, −C or D mutations (most of them germline, and in few cases by SDHA mutations), arising mainly from the stomach, with a lesser female prevalence, but histologically similar to SDHA−/IHC− GIST [15]. The SDHB+/IHC+ subgroup includes cases of NF1-mutated GIST, that are commonly intestinal, multifocal and have an IGFIR negative staining, and also sporadic KitWT/PDGFRAWT GIST, arising in the adult from any part of gastrointestinal tract [15,21,25]. In about 15% of cases of sporadic KitWT/PDGFRAWT GIST there may be an activating mutation in BRAF or, more rarely, RAS [26-28]. Taken together, cases of BRAF, RAS, or NF1 mutant GIST can be referred to as RAS-pathway (RAS-P) mutant GIST (RAS-Pmut).

In this extremely heterogeneous landscape, the clinical profile and molecular abnormalities of the small subgroup of WT GIST suitably referred to as quadruple wild-type GIST (quadrupleWT or KitWT/PDGFRAWT/SDH−/RAS−PDGFRA mut) remains undefined [29]. The aim of this study is to investigate the genomic profile of KitWT/PDGFRAWT/SDH−/RAS−PDGFRA mut GIST, by using a massively parallel sequencing and microarray approach, and compare it with the genomic profile of other GIST subtypes.

Results and discussion
Whole-Transcriptome Paired-End RNA Sequencing and copy number analysis
Whole-Transcriptome Paired-End RNA Sequencing was performed on a total of 16 GIST samples, of which 2 were KitWT/PDGFRAWT without SDH-inactivating mutations and SDHB+/IHC−/SDHA−/IHC+ (GIST_133 and GIST_127), 2 were KitWT/PDGFRAWT/SDHA*mut and SDHB−/IHC−/SDHA−/IHC− (GIST_7 and GIST_10), and 12 were Kit*mut or PDGFRA*mut. The principal component analysis showed that both GIST_133 and GIST_127 (KitWT/PDGFRAWT/SDH− and SDHB+/IHC−/SDHA−/IHC+) are characterized by a gene expression profile profoundly different from both GIST_7 and GIST_10 (KitWT/PDGFRAWT/SDHA*mut and SDHB+/IHC−/SDHA−/IHC−), while clustering in proximity of a subset of Kit*mut or PDGFRA*mut GIST (Figure 2A).

To investigate the presence of novel mutations or small ins/del in the whole coding regions of Kit and PDGFRA we analyzed whole transcriptome sequencing
Figure 1 The complexity of $KIT^{WT}/PDGFRA^{WT}$ GIST molecular biology. $KIT^{WT}/PDGFRA^{WT}$ GIST could be firstly divided into two main groups according to the SDHB immunohistochemical status: SDHB$^{IHC+}$ (including NF1-mutated GIST and sporadic $KIT^{WT}/PDGFRA^{WT}$ GIST with or without KRAS/BRAF mutations) and SDHB$^{IHC-}$ or SDH-deficient GIST. The latter could be further divided according to the SDHA immunohistochemical status: SDHB$^{IHC-}$/SDHA$^{IHC-}$ GIST (pediatric type or young adult GIST characterized by germline or somatic inactivating SDHA mutations) and SDHB$^{IHC-}$/SDHA$^{IHC+}$ GIST (including Carney-Stratakis Syndrome-related GIST, characterized by germline or somatic inactivating SDHB, −C, −D mutations, Carney Triad-related GIST that lack $SDHx$ mutations, and sporadic $KIT^{WT}/PDGFRA^{WT}$ GIST, characterized by germline or somatic inactivating SDHB, −C, −D mutations and SDHA mutations, reported in only three cases [15]). In red the subset of $KIT^{WT}/PDGFRA^{WT}$ GIST referred to as quadruple$^{WT}$ GIST ($KIT^{WT}$/PDGFRA$^{WT}$/SDH$^{WT}$/RAS-P$^{WT}$), that represent the subject of this study.

* SDHA inactivating mutations have been reported only in three cases SDHB$^{IHC-}$/SDHA$^{IHC+}$ (15).

Figure 2 Principal Component Analysis (PCA) performed on samples analyzed with RNA-seq (Figure 2A) and microarray (Figure 2B). In both cases the patients with SDHA mutations are arranged in a strongly separated cluster (yellow points), as were the $KIT^{WT}/PDGFRA^{WT}$/SDH$^{WT}$/RAS-P$^{WT}$ samples (red point) although closer to $KIT$ or PDGFR$^{A}$ mutated (respectively blue and green point).
data for single nucleotide variant (SNV) and found no private or cryptic mutations. Moreover, no NF-1, BRAF, RAS mutations were found by whole transcriptome sequencing. Therefore, the GIST from these two patients were $\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDH}^{\text{WT}}/\text{RAS-}^{\text{PWT}}$, or $\text{quadruple}^{\text{WT}}$ GIST. Analysis of deleterious mutations from whole transcriptome sequencing did not identify any known oncogenic event or shared alteration in the two patients (Additional file 1: Table S1). Copy number analysis was performed on the two $\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDH}^{\text{WT}}/\text{RAS-}^{\text{PWT}}$ GIST: GIST_133 showed no genomic imbalances, while GIST_127 harbors several macroscopic cytogenetic alterations, including loss of chromosome arms 14q and 22q frequently observed in $\text{KIT/PDGFRA}$ mutated GIST.

**Gene expression analysis**

To confirm and extend the results, whole-genome gene expression analysis by microarray was performed on 9 out 16 patients analyzed by RNAseq and an additional 20 GIST patients (1 $\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDHA}^{\text{mut}}$ GIST and 19 $\text{KIT}^{\text{mut}}$ or $\text{PDGFRA}^{\text{mut}}$ GIST). The principal component analysis confirmed that both $\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDH}^{\text{WT}}/\text{RAS-}^{\text{PWT}}$ GIST have a genetic profile significantly different from all three $\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDHA}^{\text{mut}}$ GIST, and cluster in close proximity to some $\text{KIT}^{\text{mut}}$ GIST samples (Figure 2B). Supervised gene expression analysis revealed the presence of specific genetic signatures characterizing the different molecular subgroups of GIST (Figure 3); the $\text{SDHA}^{\text{mut}}$ group showed a gene signature mainly characterized by the over-expression of $\text{IGFIR}$ (p value 2.7X10^{-11}) and of neural markers ($\text{LHX2}$, $\text{KIRREL3}$) [30], whereas as expected, all $\text{PDGFRA}^{\text{mut}}$ GIST were clearly separated from $\text{KIT}^{\text{mut}}$ GIST, especially for the expression of $\text{PDGFRA}$.

The $\text{quadruple}^{\text{WT}}$ ($\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDH}^{\text{WT}}/\text{RAS-}^{\text{PWT}}$) samples were characterized by a distinct gene expression profile (Figure 4), with 65 genes over-expressed or under-expressed (p value < 0.005) compared with all the other GIST molecular subgroups. GSEA analysis of the transcriptional profile of $\text{quadruple}^{\text{WT}}$ tumors showed enrichment of Polycomb target genes with respect to $\text{SDHA}^{\text{mut}}$ GIST, in particular of the classes of PRC2 targets (p value 0.043) and H3K27-bound genes (p value 0.021). The function of the upregulated genes was related to cell cycle progression and MAPK signaling, as exemplified by increased expression of SKP2, CDK6, FGF4, NTRK2). The $\text{quadruple}^{\text{WT}}$ GIST tumors

![Figure 3](image-url)  
**Figure 3** Representation of top-scoring genes significantly over-expressed in the four GIST classes, ($\text{KIT}^{\text{WT}}/\text{PDGFRA}^{\text{WT}}/\text{SDH}^{\text{WT}}/\text{RAS-}^{\text{PWT}}$, $\text{SDHA}^{\text{mut}}$, $\text{KIT}^{\text{mut}}$ and $\text{PDGFRA}^{\text{mut}}$), when each of them is compared with all other cases together.
are characterized by the overexpression of molecular markers (*CALCRL* and *COL22A1*) and of specific oncogenes including tyrosine and cyclin-dependent kinases (*NTRK2* and *CDK6*) and one member of the ETS-transcription factor family (*ERG*). Overexpression of *CALCRL*, *COL22A1*, *NTRK2* (TrkB) and the ETS-transcription factor *ERG* was confirmed by quantitative PCR, showing that only the *KIT*<sup>WT</sup>/*PDGFRA*<sup>WT</sup>/*SDH</sup><sup>WT</sup>/*RAS-P</sup><sup>WT</sup> GIST subgroup expressed these molecular markers and possible therapeutic targets (Figure 5). *NTRK2* protein expression level was also evaluated by Western Blot analysis and its overexpression in quadruple<sup>WT</sup> GIST was confirmed (Additional file 2: Figure S1). No mutations, gene fusions or amplifications were identified in *NTRK2* and *ERG*.

**Discussion**

The pathogenesis and underlying biology of *KIT*<sup>WT</sup>/*PDGFRA*<sup>WT</sup> with intact SDH complex (*SDHx*<sup>WT</sup>) and non-mutated RAS-pathway members (*RAS-P*<sup>WT</sup>) suitably referred to as quadruple<sup>WT</sup> GIST remains undefined. In the present study, we performed a whole genome analysis using a massively parallel sequencing approach on a total of 16 GIST cases that included 2 *KIT*<sup>WT</sup>/*PDGFRA*<sup>WT</sup>/*SDH</sup><sup>WT</sup> and 12 cases of *KIT*<sup>mut</sup> or *PDGFRA*<sup>mut</sup> GIST. Notably, we found that both cases of quadruple<sup>WT</sup> GIST had a transcriptomic profile profoundly different from both *KIT*/*PDGFRA* mutated and *SDHA*-mutated GIST, suggesting a different molecular background underlying quadruple<sup>WT</sup> GIST. Since both cases of *KIT*<sup>WT</sup>/*PDGFRA*<sup>WT</sup>/*SDH</sup><sup>WT</sup> lacked mutations of *BRAF*, RAS family members or *NF1*, the GIST of these two patients was classified *KIT*<sup>WT</sup>/*PDGFRA*<sup>WT</sup>/*SDH</sup><sup>WT</sup>/RAS-P<sup>WT</sup> or quadruple<sup>WT</sup> GIST. We further validated our data using genome wide gene expression analysis, performed on 9 cases from a previous study using massively parallel sequencing.
series that was expanded to include an additional 20 GIST cases (1 $KIT^{WT}$/$PDGFRA^{WT}$/$SDHA^{mut}$ GIST and 19 $KIT^{mut}$ or $PDGFRA^{mut}$ GIST). This larger analysis confirmed the unique gene expression signature of the two quadruple $WT$ GIST compared to $KIT$ mutant, $PDGFRA$ mutant or $SDHA$-mutant GIST. Interestingly, the gene signatures of the quadruple $WT$ GIST, which both arose in the small intestine, clustered in close proximity to a single $KIT^{mut}$ GIST sample (GIST_13). This case was a small intestine GIST of intermediate risk of relapse radically resected from a 46 year old; it harbored an exon 11 $KIT$ point mutation ($KIT$ exon 11 V559D). Our current sample size does not allow us to draw definitive conclusions, but we hypothesize that the intestinal origin of all three tumors may have influenced the gene signature. However, several other cases of small intestinal origin did not cluster near the cases of quadruple $WT$ GIST. The influence of the tissue of origin on the gene signature is consistent with the recent data by Beadling et al., who described five cluster groups among 136 GIST patients (53 $KIT^{mut}$, 12 $PDGFRA^{mut}$, 65 adult $KIT^{WT}$/$PDGFRA^{WT}$ and 7 pediatric $KIT^{WT}$/$PDGFRA^{WT}$) defined by the expression patterns of 14 target genes, that were in some cases paralleled by the location of the primary tumour [31].

Using a supervised analysis, we found four gene cluster subgroups based on $KIT$/$PDGFRA$/$SDH$-mutational status. Due to the rarity of $RAS-P$ mutated GIST, we did not have any cases suitable for these genomic studies. Consistent with previous reports, $KIT^{WT}$/$PDGFRA^{WT}$/$SDHA^{mut}$ GIST over-expressed $IGF1R$, further confirming the potential role of this receptor as a target or diagnostic marker for this specific molecular subgroup [18-21]. Moreover, as already described, the gene signature of $KIT^{WT}$/$PDGFRA^{WT}$/$SDHA^{mut}$ GIST was largely characterized by the expression of neural-commitment transcription markers, in support of the theory that this subgroup may have a different cellular origin or may derive from interstitial cells of Cajal (ICCs) during a different differentiation step, such as from precursors of ICCs [30]. Notably, both quadruple $WT$ GIST had a distinct gene expression signature that was separated from the $KIT^{WT}$/$PDGFRA^{WT}$/$SDHA^{mut}$ GIST. Amongst the differentially expressed genes, it is interesting to note the over-expression of $CALCRL$, a G protein-coupled receptor that acts as a receptor for adrenomedullin and calcitonin gene-related peptide (CGRP), and is strongly expressed by several vascular tumours and types of gliomas [32-35]. Also of interest, we found over-expression of $COL22A1$, a member of the collagen protein family which specifically localizes to tissue junctions and acts as a cell adhesion ligand for skin epithelial cells and fibroblasts [36]. Taken together, these findings may suggest the potential role of $CALCRL$ and $COL22A1$ as diagnostic markers for the identification of this GIST subgroup. This would need to be validated in a larger series of GIST.

We found that both quadruple $WT$ GIST, in comparison with the other samples, strongly expressed several
Over-expression of ERG and NTRK2 (TrkB). This was confirmed by quantitative PCR. ERG is a well-known member of the erythroblast transformation-specific (ETS) family of transcription factors, which function as transcriptional regulators [37]. ETS proteins are regulated by the mitogenic (RAS/MAPK) signalling transduction pathway, and play an important role in cell differentiation, proliferation, apoptosis and tissue remodelling [38]. There is evidence for an oncogenic role of ERG and the other ETS transcription factors in many human cancers, including sarcomas, prostate cancer, and acute myeloid leukemia, in most cases via chromosomal translocations [39-41]. More recently, it has been shown that the IHC detection of ERG is also of interest, as NTRK2 helps regulated neuronal cell function, including synaptic plasticity, differentiation, growth, survival, and motility [45]. It has also been shown that Trks regulate important processes in non-neuronal cells, contributing to the pathogenesis of several kinds of cancer, such as medullary thyroid carcinoma, prostate cancer, non-small cell lung cancer, head and neck squamous cell carcinoma and pancreatic cancer; in addition to tumors of neural origin [46-51]. Given the relevant biological role played by Trks in cancer, different small molecule inhibitors have been developed and evaluated both in mono-therapy and in combination with chemotherapy in phase 1 and 2 clinical trials [52-58].

To our knowledge, the over-expression of ERG and TrkB in GIST has not been previously reported. However, it is well known that ETVI, another member of ETS family, is highly expressed in GIST and certain subsets of ICC. ETVI expression plays an important role in regulating the growth of KIT mutant GIST cell lines [59]. On the basis of our results, the overexpression of ERG and TrkB seems to be a unique feature of the quadruple WT GIST, suggesting that it could play a relevant role in the pathogenesis of this subset of GIST. To translate these observations into clinical practice, the overexpression of both molecules could be investigated as diagnostic markers of quadruple WT GIST.

Conclusions

In conclusion, we report for the first time an integrated genomic picture of the quadruple WT GIST, using massively parallel sequencing and gene expression analyses, and have identified a unique subset of GIST among the family of the KIT/PDGFRA WT GIST [60]. The frequency of this GIST subset amongst the family of GIST will need to be defined in future studies as well as any unique clinical-pathological features of this GIST subset, including response to conventional GIST medical therapy. In addition, ongoing studies of ICC developmental biology may help identify the “normal” precursor cells that give rise to this unique GIST subgroup.

Methods

This study was approved by the institutional review board of Azienda Ospedaliero-Universitaria Policlinico S.Orsola-Malpighi, Bologna, Italy (approval number 113/2008/U/Tess). All patients provided written informed consent.

Patients and tumor samples

Fresh tissue specimens of GIST from 36 patients were collected during the surgical operation, snap-frozen in liquid nitrogen and stored at ~80°C until analysis. Patient’s characteristics are listed in Table 1.

Whole-Transcriptome Paired-End RNA Sequencing was performed on 16 GIST, including 2 KIT WT/PDGFRA WT GIST patients without SDH-inactivating mutations (GIST_133 and GIST_127), 2 KIT WT/PDGFRA WT GIST patients harbouring SDHA-mutations (GIST_7 and GIST_10), and 12 KIT or PDGFRA mutated GIST patients (7 harboured exon 11 KIT mutations and 5 harboured exon 18 PDGFRA mutations).

Whole-genome gene expression analysis was performed on 9 of the above 16 GIST and extended to include an additional 20 GIST: 1 KIT WT/PDGFRA WT/SDHA mut GIST and 19 KIT or PDGFRA mutated GIST, of which 13 harboured KIT mutations (12 in exon 11 and 2 in exon 9), and 5 harboured PDGFRA mutations (2 in exon 12, 1 in exon 14 and 2 in exon 18).

SDH status

SDH protein expression status was evaluated by both immunohistochemistry (IHC) of SDHB and SDH subunits sequencing. IHC was performed on 4-μm sections of FFPE GIST tumor samples. Rabbit polyclonal anti-SDHB (HPA002868, Sigma-Aldrich, St Louis, MO, USA, 1:800) antibody was used. The sections were deparaffinized, rehydrated, and subjected to the appropriate antigen retrieval treatment (SDHB: microwave heating in citrate buffer pH 6.0 at 100 1C for 40 min). After cooling at room temperature, the activity of endogenous peroxidises was inhibited using methanol/H2O2 (0.5% v/v) for 20 min. The sections were then washed in phosphate-buffered saline (PBS, pH 7.2-7.4) and incubated with the specific primary antibody overnight at room temperature. After that, the sections were washed in PBS and treated using the Novolink Polymer Detection System (Novoceastra, Newcastle upon Tyne, UK) according to the manufacturer’s instructions. Liver tissues (for SDHB) were used as positive controls. These tissues showed strong granular staining in the cytoplasm and mitochondria with both of the antibodies.
SDHA gene exons [1-15], SDHB gene exons [1-8], SDHC (exon 1–6) and SDHD (exon 1–4) were sequenced on fresh-frozen tumor specimens of KITWT/PDGFRAWT GIST patients by Sanger Sequencing method. DNA was extracted by the QIAmp DNA Mini kit (Qiagen, Milan, Italy) in accordance with manufacturer's directions. Each exon was amplified with Polymerase Chain Reaction (PCR) amplification using specific primer pairs designed with Primer Express 3.0 Software (Applied Biosystem) to amplify exons but not SDHA pseudo-genes located on chromosomes 3 and 5. Then, PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Milan, Italy) and sequenced on both strands using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Sanger

### Table 1 Patient's characteristic

| ID    | Sex | Array | RNAseq | Age | Site      | Disease status at diagnosis | KIT/PDGFRASDH mutational status                     |
|-------|-----|-------|--------|-----|-----------|-----------------------------|-----------------------------------------------------|
| GIST_133 | M   | X     | X      | 57  | Duodenum  | Localized                   | WT                                                  |
| GIST_127 | F   | X     | X      | 63  | Ileum     | Localized                   | WT                                                  |
| GIST_07  | F   | X     | X      | 27  | Stomach   | Metastatic                  | SDHA exon 9 p.S384X                                  |
| GIST_10  | M   | X     | X      | 29  | Stomach   | Metastatic                  | SDHA exon 2 p.R31X; SDHA exon 13 p.R589W             |
| GIST_188 | F   | X     | X      | 57  | Duodenum  | Metastatic                  | KIT exon 11 p.N564-L576 del + KIT exon 17 p.N822K   |
| GIST_174 | M   | X     | X      | 59  | Stomach   | Metastatic                  | KIT exon 11 p.N564_L576 del + KIT exon 17 p.N822K   |
| GIST_131 | M   | X     | X      | 58  | Ileum     | Localized                   | KIT exon 11 p.V569_Y578 del                          |
| GIST_11  | M   | X     | X      | 65  | Stomach   | Localized                   | KIT exon 11 p.S57-558 del                           |
| GIST_134 | F   | X     | X      | 65  | Stomach   | Localized                   | KIT exon p.V559D                                    |
| GIST_124 | M   | X     | X      | 70  | Stomach   | Localized                   | KIT exon 11 p.1765-1766 ins                          |
| GIST_150 | F   | X     | X      | 55  | Stomach   | Localized                   | KIT exon 11 p.E551_E554 del                          |
| GIST_165 | M   | X     | X      | 50  | Stomach   | Localized                   | PDGFRAX exon 18 p.D842V                              |
| GIST_136 | M   | X     | X      | 76  | Stomach   | Localized                   | PDGFRAX exon 18 p.D842V                              |
| GIST_140 | F   | X     | X      | 45  | Stomach   | Localized                   | PDGFRAX exon 18 p.D842V                              |
| GIST_141 | M   | X     | X      | 68  | Stomach   | Localized                   | PDGFRAX exon 18 p.D842V                              |
| GIST_138 | F   | X     | X      | 75  | Stomach   | Localized                   | PDGFRAX exon 18 p.D842V                              |
| GIST_02  | F   | X     | X      | 85  | Stomach   | Localized                   | KIT exon 11 p.V560D                                  |
| GIST_04  | M   | X     | X      | 79  | Stomach   | Localized                   | KIT exon 9 p.A502-S503 ins                           |
| GIST_05  | M   | X     | X      | 68  | Stomach   | Localized                   | PDGFRAX exon 12 p.SPDGHE566-571RIQ                   |
| GIST_08  | M   | X     | X      | 62  | Stomach   | Localized                   | KIT exon 11 p.V559D                                  |
| GIST_09  | M   | X     | X      | 54  | Stomach   | Localized                   | KIT exon 11 TLQPYDHKWEFP 574-585 ins at PS85        |
| GIST_12  | F   | X     | X      | 66  | Stomach   | Localized                   | PDGFRAX exon 14 p.K646E                              |
| GIST_13  | M   | X     | X      | 46  | Small intestine | Localized                   | KIT exon 11 p.V559D                                  |
| GIST_14  | M   | X     | X      | 56  | Stomach   | Localized                   | KIT exon 11 p.WKSS7-S558del                           |
| GIST_15  | F   | X     | X      | 64  | Stomach   | Localized                   | PDGFRAX exon 18 DIMH p.B42-845 DIMH del              |
| GIST_16  | F   | X     | X      | 62  | Stomach   | Localized                   | KIT exon 11 p.L576P                                  |
| GIST_20  | M   | X     | X      | 38  | Small intestine | Metastatic                   | KIT exon 11 del MYEQW552-557 Z + KIT exon 18 A829P |
| GIST_22  | F   | X     | X      | 76  | Stomach   | NA                         | PDGFRAX exon 18 p.D842V                              |
| GIST_23  | F   | X     | X      | 47  | Stomach   | NA                         | KIT exon 11 p.V559D                                  |
| GIST_24  | F   | X     | X      | 18  | Stomach   | Metastatic                  | SDHA exon 8 p.L349R fs*11                            |
| GIST_26  | M   | X     | X      | 49  | Stomach   | Localized                   | PDGFRAX exon 12 p.V561D                              |
| GIST_121 | M   | X     | X      | 71  | Stomach   | Localized                   | KIT exon 11 p.V559D                                  |
| GIST_125 | F   | X     | X      | 48  | Stomach   | Localized                   | KIT exon 11 p.W557R                                  |
| GIST_129 | M   | X     | X      | 59  | Stomach   | Localized                   | KIT exon11 p.Y553_V559 delins L                      |
| GIST_130 | F   | X     | X      | 79  | Stomach   | Localized                   | KIT exon 9 p.A502-Y503 ins                           |
| GIST_135 | F   | X     | X      | 61  | Stomach   | Localized                   | KIT exon 11 p.W557-E561 del                          |
sequencing was performed on ABI 3730 Genetic Analyzer (Applied Biosystems).

**Whole-transcriptome paired-end RNA sequencing**

Total RNA was extracted from tumor specimens with RNeasy Mini Kit (Qiagen, Milan, Italy), then cDNA libraries were synthesized from 250 ng total RNA with TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA) according to the manufacturer's recommendations. Sequencing by synthesis was performed on HiScanSQ sequencer (Illumina) at 75 bp in paired-end mode. Whole-transcriptome sequencing yielded an average of 61 million mapped reads/patient, thus reaching an average coverage of 44X. Two $SDHA^{mut}$ tumor specimens were previously analyzed by whole transcriptome sequencing at the Genome Sciences Centre (Vancouver, Canada) [9].

**Bioinformatic analysis**

After demultiplexing and FASTQ generation (both steps performed with Casava1.8, an application software specifically developed by Illumina), the paired-end reads quality were analyzed with the function fastx_quality_stats (part of FASTX Toolkit available at http://hannon-lab.cshl.edu/fastx_toolkit/index.html). Based on these results we decided to trim each read of each sample at 74 bp in order to maximize sequence quality. The paired-end reads were mapped with the pipeline TopHat/Bowtie [61] on human reference genome HG19, collected from UCSC Genome Browser (http://www.genome.ucsc.edu/). After the alignment procedure the BAM file obtained was manipulated with Samtools [62] in order to remove the optical/PCR duplicate, to sort and to index it.

The analysis of gene expression was performed in two steps: 1) the function htsq-cound (Python package HTSeq) [63] was adopted to count the number of reads mapped on known genes, included in the Ensembl release 72 annotation features (http://www.ensembl.org/); 2) the differential expressed genes were evaluated using the R-Bioconductor package edger [64]. DeFuse, ChimeraScan and FusionMap packages were used to detect chimeric transcripts from RNA-seq data.

**Gene expression analysis**

RNA was extracted using RNeasy Mini Kit (Qiagen), quality-controlled and labeled as directed by the Affymetrix expression technical manual before hybridization to U133Plus 2.0 arrays. Gene expression data were quantified by the RMA algorithm, filtered and analyzed with supervised techniques by Limma modified t-test for the detection of differentially expressed genes. Differential expressed genes hierarchical clustering and Principal Component Analysis (PCA) were performed with Multiple Array Viewer (MEV available at http://www.tm4.org/mev.html).

The same software was used to represent the data in the Figure 3 and Figure 4. Gene expression data of $KIT/ PDGFRA^{mut}$-mutated and $SDHA^{mut}$-mutated samples were previously reported [30].

**Copy number analysis**

Genomic DNA was labelled and hybridized to SNP array Genome Wide SNP 6.0 (Affymetrix) following manufacturer's instructions. Quality control was performed by Contrast QC and MAPD calculation. Copy number analysis was performed by Genotyping Console and visualized with Chromosome Analysis Suite (ChAS) Software (Affymetrix). Hidden Markov Model algorithm was used to detect amplified and deleted segments with stringent parameters. To control for hyperfragmentation adjacent segments separated by < 50 probes were combined into one single segment, and only segments > 100 probes were considered.

**Quantitative PCR (qPCR)**

Total RNA was reverse transcribed using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Monza, Italy) with oligo-dT primers, according to the manufacturer's guidelines. Gene-specific primers were designed with Primer Express 3.0 Software (Applied Biosystems) and qPCR was performed using FastStart Sybr Green (Roche) on the LightCycler 480 apparatus (Roche). DDCt method was used to quantify gene product levels relative to the GAPDH and ATP5B housekeeping genes. Significance was estimated by the Student’s $t$ test: * $p$-value < 0.05; ** $p$-value < 0.01, *** $p$-value < 0.01.

**Western blot**

Protein expression of $NTRK2$ was evaluated on 2 $KIT^{WT}/PDGFRA^{WT}/SDH^{WT}/RAS-P^{WT}$ GIST and 8 $KIT$ or $PDGFRA$ or $SDH$ mutant GISTs, of which fresh-frozen tissues were available. Tissue were disrupted in RIPA buffer (Sigma-Aldrich) supplemented with proteases inhibitors and lysed for 1 h with gentle agitation at 4°C. Lysates were centrifuged at 13,000 x g for 15 min at 4°C and supernatants were stored at −80°C. Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL). Twenty micrograms of protein were resolved on a 8% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes. Non-specific binding sites were blocked by incubation in blocking buffer (PBS containing 0.1% Tween-20 with 5% w/v milk) for 1 h at room temperature. Membranes were incubated overnight at 4°C, with the following primary antibodies: rabbit polyclonal $TRKB$ antibody (ab18987 Abcam 1:500), and rabbit polyclonal $\beta$-Tubulin antibody (sc-9104 Santa Cruz Biotechnology, Santa Cruz, CA, 1:500). Then, membranes were washed and incubated with peroxidase conjugate secondary antibodies for 1 h at
Nannini et al. BMC Cancer 2014, 14:685
http://www.biomedcentral.com/1471-2407/14/685

Page 10 of 12

room temperature. Antigens were revealed using Enhanced Chemiluminescence Reaction (ECL Select, Amersham Pharmacia Biotech, Les Ulis, France).

Nomenclature

KIT\[^{WT}\] No mutations of KIT
PDGFRA\[^{WT}\] No mutations of PDGFRA
SDH\[^{WT}\] No abnormalities of SDHA/B/C/D protein expression and/or gene mutation
SDHA\[^{HIC}\] – No expression of SDHA protein
SDHA\[^{HIC}\] + Normal expression of SDHA protein
SDHB\[^{HIC}\] – No expression of SDHB protein
SDHB\[^{HIC}\] + Normal expression of SDHB protein
SDHA\[^{mut}\] Mutation of SDHA protein (homozygous or compound heterozygote)
SDHB\[^{mut}\] Mutation of SDHB protein (homozygous or compound heterozygote)
SDHC\[^{mut}\] Mutation of SDHC protein (homozygous or compound heterozygote)
SDHD\[^{mut}\] Mutation of SDHD protein (homozygous or compound heterozygote)

Additional files

Additional file 1: Table S1. NTRK2 protein overexpression in KIT\[^{WT}\]/PDGFRA\[^{WT}\]/SDH\[^{WT}\]/RAS-P\[^{WT}\] GIST. Western blot immunostaining of NTRK2 was performed on proteins extracted from two quadruplet\[^{WT}\] GIST and from eight PDGFRA or KIT or SDH mutated GIST. HL-60 cell line protein extract was used as positive control.

Additional file 2: Figure S1. NTRK2 protein overexpression in KIT\[^{WT}\]/PDGFRA\[^{WT}\]/SDH\[^{WT}\]/RAS-P\[^{WT}\] GIST. Western blot immunostaining of NTRK2 was performed on proteins extracted from two quadruplet\[^{WT}\] GIST and from eight PDGFRA or KIT or SDH mutated GIST. HL-60 cell line protein extract was used as positive control.

Abbreviations
gCGRP: Calcitonin gene-related peptide; CSS: Carney-Stratakis Syndrome; CT: Carney Triad; DOG1: Discovered on gastrointestinal stromal tumours 1; ETS: Erythroid transformation-specific; GIST: Gastrointestinal stromal tumors; ICCs: Cells of Cajal; IGF1R: Insulin growth factor receptor 1; IHC: Immunohistochemistry; NFI: Neurofibromatosis type 1; PDGFRA: Platelet-derived growth factor receptor alpha; RAS-P: RAS-pathway; SDH: Succinate dehydrogenase; SM: Single nucleotide variant; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

MN: have made substantial contributions to conception and design of the study, interpretation of data and drafted the manuscript; AA: carried out the molecular genetic studies, the sequence alignment and have been involved in drafting the manuscript. MU: carried out the molecular genetic studies, the sequence alignment and have been involved in drafting the manuscript. V: carried out the bioinformatic analysis and interpretation of data and have been involved in drafting the manuscript. DC: carried out the pathological analysis and the collection of samples, MCH: have been involved in revising the manuscript critically for important intellectual content and have given final approval of the version to be published. CLC: have been involved in revising the manuscript critically for important intellectual content and have given final approval of the version to be published. GB: have been involved in revising the manuscript critically for important intellectual content and have given final approval of the version to be published. MAP: have made substantial contributions to conception and design of the study, interpretation of data and drafted the manuscript; All authors read and approved the final manuscript.

Acknowledgments

All staff of Bologna GIST Study Group: Annalisa Altimari, Claudio Cecarelli, Paolo Castellucci, Fausto Catena, Monica Di Battista, Massimo Del Gaudio, Valerio Di Scioscio, Stefano Fanti, Michelangelo Fiorentino, Pietro Fusaroli, Lidia Gatto, Franco W. Grigioni, Elisa Grupponi, Alessandra Maleddu, Maria Caterina Pallotti, Antonino Daniele Pinta, Paola Tommassetti, Maurizio Zompatori.

Funding

The present work was done with a financial contribution by Novartis Oncology, Italy, and with funds by My First Grant 2013, AIRC 2013.

Author details

1Department of Specialized, Experimental and Diagnostic Medicine, Sant’Orsola-Malpighi Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy. 2“Giorgio Prodi” Cancer Research Center, University of Bologna, Bologna, Italy. 3Pathology Unit, S. Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy. 4Portland VA Medical Center and Knight Cancer Institute, and Division of Hematology and Oncology, Oregon Health & Science University Portland, Portland, OR, USA. 5Department of Pathology and Knight Cancer Institute, Oregon Health & Science University, Portland, OR, USA. 6Transplant, General and Emergency Surgery Department, S. Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy.

Received: 23 January 2014 Accepted: 17 September 2014 Published: 20 September 2014

References

1. Corless CL, Fletcher JA, Heinrich MC: Biology of gastrointestinal stromal tumors. J Clin Oncol 2004, 22:3813–3825.
2. Janeway KA, Pappo AS: Pediatric gastrointestinal stromal tumor. Hematol Oncol Clin North Am 2009, 23:35–34.
3. Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McClellan LS, Chen CJ, Van den Abbeele AD, Druker BJ, Kiese B, Eisenberg B, Roberts PJ, Singer S, Fletcher CD, Silberman S, Dimitrjevic S, Fletcher JA: Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003, 21:4342–4349.
4. Debiec-Rychter M, Sciorti R, Le Cesne A, Schlemmer M, Hohenberger P, van Oosterom AT, Bly JB, Levyra S, Stul M, Casali PG, Zalcberg J, Verweij J, Van Giabbeke M, Hagermeier A, Judson I, EORTC Soft Tissue and Bone Sarcoma Group; Italian Sarcoma Group; Australian Gastrointestinal Trials Group: Kit mutations and dose selection for imatinib in patients with advanced gastrointestinal stromal tumours. Eur J Cancer 2006, 42:1093–1103.
5. Heinrich MC, Owzar K, Corless CL, Hollis D, Borden EC, Fletcher CD, Ryan CW, von Mehren M, Blanke CD, Rankin C, Benjamin RS, Bramwell VH, Demetri GD, Bentagolli MM, Fletcher JA: Correlation of kinase genotype and clinical outcome in the North American Intergroup Phase III Trial of imatinib mesylate for treatment of advanced gastrointestinal stromal tumor: CALGB 150105 Study by Cancer and Leukemia Group B and Southwest Oncology Group. J Clin Oncol 2008, 26:5360–5367.
6. Gill AJ, Chou A, Vilain R, Clarkson A, Lui M, Jin R, Tobias V, Samra J, Goldstein S, Smith C, Sioson L, Parker N, Smith RC, Sywak M, Sudhir SB, Wyatt JM, Robinson BG, Eckstein RP, Benn DE, Clifton-Bingham J: Immunohistochemistry for SDHB divides gastrointestinal stromal tumors (GISTs) into 2 distinct types. Am J Surg Pathol 2010, 34:636–644.
7. Miettinen M, Wang ZF, Sarlomo-Rikala M, Oshcu C, Rutkowski P, Lasota J: Succinate dehydrogenase-deficient GISTs: a clinicopathologic, immunohistochemical, and molecular genetic study of 66 gastric GISTs with predilection to young age. Am J Surg Pathol 2011, 35:1712–1721.
8. Janeway KA, Kim SY, Ledith M, Nosik V, Rustin P, Gaal J, Daha PL, Liegl B, Ball ER, Raygada M, Lai AH, Kelly L, Hornick JL, NH Pediatic and Wild-Type GIST Clinic; O’Sullivan M, de Krrijger RR, Dinjing WN, Demetri GD, Antonescu CR, Fletcher JA, Helman I, Stratakis CA: Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. Proc Natl Acad Sci U S A 2011, 108:314–318.
9. Pantaleo MA, Astolfi A, Indrio V, Moore R, Thiessen N, Heinrich MC, Groccchi C, Santini D, Catena F, Formica S, Martelli PL, Casadio R, Pession A, Bisceglia G: SDHA loss-of-function mutations in KIT/PDGFRA wild-type gastrointestinal stromal tumors identified by massively parallel sequencing. J Natl Cancer Inst 2011, 103:985–987.

10. Pantaleo MA, Nanni M, Astolfi A, Bisceglia G: GIST Study Group. Bologna: A distinct pediatric-type gastrointestinal stromal tumor in adults: potential role of succinate dehydrogenase subunit A mutations. Am J Surg Pathol 2011, 35:1750–1752.

11. Mennell HD, Hallier-Neelsen M, Hagner S, Benes L: Two novel cell specific receptor proteins, CRLR and CD 117 in human gingival tumours. Clin Neuropathol 2006, 25:107–114.

12. Pantaleo MA, Nanni M, Corless CL, Heinrich MC: Quadruple wild-type (WT) GIST: defining the subset of GISTs that lack abnormalities of KIT, PDGFRA, SDH and the RAS signalling pathway. Cancer Med 2014. in press.

13. Mennell HD, Hallier-Neelsen M, Hagner S, Benes L: The immunohistochemical expression of calcitonin receptor-like receptor (CRLR) in human gingivae. J Clin Pathol 2004, 57:172–176.

14. Koch M, Schulze J, Hansen U, Ashworth T, Kristge VR, Brunkner P, Burgos RE, Bruckner-Tuderman L: A novel marker of tissue junctions, collagen XXII. J Biol Chem 1994, 269:1430–1430.

15. Montgomery RS, Goldstein AM, Ransome KE, Buhler DS, Fetsch JF: Expression of calcitonin, calcitonin gene-related peptide, adrenomedullin and amylin: homologous peptides, separate receptors and overlapping biological actions. Eur J Endocrinol 1995, 133:17–20.

16. Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ, Denny CT: IMMORTAL: A novel marker of tissue junctions. Eur J Hum Genet 2008, 16:1413–1416.

17. Mennell HD, Hallier-Neelsen M, Hagner S, Benes L: Acute myeloid leukemia: a central role for the ETS factor ERG. Int J Biochem Cell Biol 2011, 43:1413–1416.

18. Mennell HD, Hallier-Neelsen M, Hagner S, Benes L: Acute myeloid leukemia: a central role for the ETS factor ERG. Int J Biochem Cell Biol 2011, 43:1413–1416.

19. Mennell HD, Hallier-Neelsen M, Hagner S, Benes L: Acute myeloid leukemia: a central role for the ETS factor ERG. Int J Biochem Cell Biol 2011, 43:1413–1416.
receptors in medullary thyroid carcinoma development and progression. Proc Natl Acad Sci U S A 1999, 96:4540–4545.

48. Satoh F, Mimata H, Nomura T, Fujita Y, Shin T, Sakamoto S, Hamada Y, Nomura Y. Autocrine expression of neurotrophins and their receptors in prostate cancer. Int J Urol 2001, 8:528–534.

49. Harada T, Yatabe Y, Takeshita M, Koga T, Yano T, Wang Y, Giaccone G. Role and relevance of TrkB mutations and expression in non-small cell lung cancer. Clin Cancer Res 2011, 17:2638–2645.

50. Kupferman ME, Jiffar T, El-Naggar A, Yilmaz T, Zhou G, Xie T, Feng L, Wang J, Holsinger FC, Yu D, Myers JN. TrkB induces EMT and has a key role in invasion of head and neck squamous cell carcinoma. Oncogene 2010, 29:2047–2059.

51. Scibas GM, Fujikawa S, Schmidt C, Li Z, Frederick WA, Yang W, Yokoi K, Evans DB, Abbruzzese JL, Hess KR, Zhang W, Fidler IJ, Chiao PJ. Overexpression of tropomyosin-related kinase B in metastatic human pancreatic cancer cells. Clin Cancer Res 2005, 11:440–449.

52. Camoratto AM, Jani JP, Angeles TS, Maroney AC, Sanders CV, Murakata C, Neff NT, Vaught JL, Isaacs JT, Dionne CA. CEP-751 inhibits TRK receptor tyrosine kinase activity in vitro exhibits anti-tumor activity. Int J Cancer 1997, 72:673–679.

53. Miknyoczki SJ, Dionne CA, Klein-Szanto AJ, Ruggeri BA. The novel Trk receptor tyrosine kinase inhibitor CEP-701 (KT-5555) exhibits antitumor efficacy against human pancreatic carcinoma (Panc1) xenograft growth and in vivo invasiveness. Ann N Y Acad Sci 1999, 880:252–262.

54. Evans AE, Kisselbach KD, Yamashiro DJ, Ikegaki N, Camoratto AM, Dionne CA, Brodeur GM. Antitumor activity of CEP-751 (KT-6587) on human neuroblastoma and medulloblastoma xenografts. Clin Cancer Res 1999, 5:3594–3602.

55. Strock CJ, Park JI, Rosen M, Dionne C, Ruggeri B, Jones-Bolin S, Denneau SR, Ball DW, Nelkin BD. CEP-701 and CEP-751 inhibit constitutively activated RET tyrosine kinase activity and block medullary thyroid carcinoma cell growth. Cancer Res 2003, 63:5559–5563.

56. Marshall JL, Kindler H, Deeken J, Bhargava P, Vogelzang NJ, Rzvi N, Luthala T, Boylan S, Dordal M, Robertson P, Hawkins MJ, Ratain MJ. Phase I trial of orally administered CEP-701, an onco-neurotrophin receptor-linked tyrosine kinase inhibitor. Invest New Drugs 2005, 23:31–37.

57. Wang T, Lamb ML, Scott DA, Wang H, Block MH, Lyne PD, Lee JW, Davies AM, Zhang HJ, Zhu Y, Gu F, Han Y, Wang B, Mohr PJ, Kaus RJ, Josey JA, Hoffmann E, Theiss K, Macintyre T, Wang H, Omer CA, Yu D. Identification of 4-aminopyrazolopyrimidines as potent inhibitors of Trk kinases. J Med Chem 2008, 51:4672–4684.

58. Chan E, Mullerin D, Rothenberg M, Helen KD, Lockhart AC, Thomas J, Berlin J. A phase I trial of CEP-701 + gemcitabine in patients with advanced adenocarcinoma of the pancreas. Invest New Drugs 2008, 26:241–247.

59. Chi P, Chen Y, Zhang L, Guo X, Wongvipat J, Shamu T, Fletcher JA, Dewell S, Maki RG, Zheng D, Antonescu CR, Allis CD, Sawyers CL. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. Nature 2010, 467:849–853.

60. Nannini M, Biasco G, Astolfi A, Fanalea MA. An overview on molecular biology of KIT/ PDGFRα wild-type (WT) gastrointestinal stromal tumours (GIST). J Med Genet 2013, 50:663–661.

61. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009, 25:1105–1111.

62. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Hornier N, Marth G, Abecasis G, Durbin R. 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009, 25:2078–2079.

63. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010, 11:R106.

64. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010, 26:139–140.