qPCR in gastrointestinal stromal tumors: Evaluation of reference genes and expression analysis of KIT and the alternative receptor tyrosine kinases FLT3, CSF1-R, PDGFRB, MET and AXL

Jana Fassunke, Marie-Christine Blum, Hans-Ulrich Schildhaus, Marc Zapatka, Benedikt Brors, Helen Künstlinger, Reinhard Büttner, Eva Wardelmann, Sabine Merkelbach-Bruse

Abstract

Background: Gastrointestinal stromal tumors (GIST) represent the most common mesenchymal tumors of the gastrointestinal tract. About 85% carry an activating mutation in the KIT or PDGFRA gene. Approximately 10% of GIST are so-called wild type GIST (wt-GIST) without mutations in the hot spots. In the present study we evaluated appropriate reference genes for the expression analysis of formalin-fixed, paraffin-embedded and fresh frozen samples from gastrointestinal stromal tumors. We evaluated the gene expression of KIT as well as of the alternative receptor tyrosine kinase genes FLT3, CSF1-R, PDGFRB, AXL and MET by qPCR. wt-GIST were compared to samples with mutations in KIT exon 9 and 11 and PDGFRA exon 18 in order to evaluate whether overexpression of these alternative RTK might contribute to the pathogenesis of wt-GIST.

Results: Gene expression variability of the pooled cDNA samples is much lower than the single reverse transcription cDNA synthesis. By combining the lowest variability values of fixed and fresh tissue, the genes POLR2A, PPIA, RPLPO and TFRC were chosen for further analysis of the GIST samples. Overexpression of KIT compared to the corresponding normal tissue was detected in each GIST subgroup except in GIST with PDGFRA exon 18 mutation. Comparing our sample groups, no significant differences in the gene expression levels of FLT3, CSF1R and AXL were determined. An exception was the sample group with KIT exon 9 mutation. A significantly reduced expression of CSF1R, FLT3 and PDGFRB compared to the normal tissue was detected. GIST with mutations in KIT exon 9 and 11 and in PDGFRA exon 18 showed a significant PDGFRB downregulation.

Conclusions: As the variability of expression levels for the reference genes is very high comparing fresh frozen and formalin-fixed tissue there is a strong need for validation in each tissue type. None of the alternative receptor tyrosine kinases analyzed is associated with the pathogenesis of wild-type or mutated GIST. It remains to be clarified whether an autocrine or paracrine mechanism by overexpression of receptor tyrosine kinase ligands is responsible for the tumorigenesis of wt-GIST.
Background

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract and are characterized by the expression of the KIT receptor (stem cell factor receptor, CD117) and to a lesser extent of PDGFRA (platelet derived growth factor receptor alpha), representing two closely related receptor tyrosine kinases (RTK) [1,2]. The majority of GIST shows oncogenic mutations either in KIT or PDGFRA [3,4]. Mainly, mutations in exon 9 or 11 of the KIT gene or in exon 18 of PDGFRA lead to ligand independent, constitutive activation of the kinase function [5]. About 60% of all GIST carry an exon 11 mutation of KIT which encodes the juxtamembrane domain of the receptor possessing an autoinhibitory function [6,7]. Less common mutations in PDGFRA (~10%) are detected in GIST that often display gastric location and epithelioid morphology [2].

In a minority of cases (10-15%) no mutations in the known KIT or PDGFRA hot spots are detected although these tumors express the KIT protein. This subgroup is called wild type GIST (wt-GIST) and comprises tumors in pediatric patients, in patients affected by the Carney triad, neurofibromatosis type 1 (NF1) associated GIST and a subset of sporadic adult GIST [8-11]. The pathogenetic mechanisms underlying wt-GIST are poorly understood and there is limited benefit of imatinib therapy in these patients [12]. Therefore the identification of additional genetic factors contributing to the pathogenesis of GIST may help to find new concepts of individualized therapy.

Recently, the BRAF mutation p.V600E was found in 4-13% of wt-GIST [13-15]. For another subgroup of wt-GIST including pediatric tumors, a strong IGF1R expression combined partly with gene amplification was described [16-18]. Two other alternative RTK probably involved in the pathogenesis of GIST are AXL and MET. Both kinases have been shown to be upregulated in GIST resistant to treatment [19]. AXL is a member of the Ufo/AXL subfamily and activates the same signaling pathway as KIT. The tyrosine kinase domain of MET is mutated in sporadic papillary renal carcinomas. Some mutations in the MET gene are located in codons homologous to those in KIT and it is suggested that these missense mutations lead to constitutive activation of the MET protein [20]. To develop additional therapy approaches it would be of interest to know whether this RTK also plays a role in wt-GIST.

Besides KIT and PDGFRA, CSF1R (colony stimulating factor 1 receptor), FLT3 (fms like tyrosine kinase 3) and PDGFRB (platelet derived growth factor receptor β) belong to the same family of type III RTK. These five tyrosine kinases show a homologous structure and a comparable function in activation, proliferation and suppressing apoptosis [21-23].

Aberrant expression and mutations in either CSF1R, FLT3 and PDGFRB or their ligands have been described in several malignant diseases. Overexpression of CSF1R is found in epithelial tumors such as breast and ovarian cancer [24]. The translocation t(1;2) of its ligand CSF1 leads to the development of the tenosynovial giant cell tumor [25].

Aberrently expressed FLT3 is observed at high levels in a spectrum of hematologic malignancies [26]. Additionally, in AML an internal tandem duplication in the transmembrane domain of FLT3 was identified which leads to constitutive activation of its kinase domain. It seems that this mutation is not present or very rare in GIST [27].

PDGFRB is overexpressed in malignant peripheral nerve sheath tumors (MPNST) and chordomas [28,29].

To analyse expression profiles of certain tumors for research and diagnostic purposes, qPCR (quantitative PCR) is frequently applied because of its reproducibility and high sensitivity [30,31]. This method is based on the normalization of the target gene expression on stably expressed internal reference genes. A major challenge is the application of suitable reference genes which have to be tested and verified under defined experimental conditions [32,33]. Ideal reference genes have to be non-regulated, stable and not affected by biological or experimental conditions. The target gene is amplified together with the reference gene in order to minimize experimental variability concerning reverse transcription enzymatic efficiencies, PCR efficiency, amount of starting material and differences between human tissues. The reference gene and the target gene should have very robust and stable expression profiles to ensure accurate normalization and interpretation of results. The most stable expressed gene from a set of genes can be identified by geNorm [34], a software program which additionally provides the number of genes required to calculate a robust normalization factor based on the geometric mean of these genes.

Typical reference genes regulate basic and ubiquitous cellular functions and are responsible for the cellular maintenance, e.g. GAPDH or β-actin. However, these commonly used reference genes vary considerably in different tissue types or under different experimental conditions [35,36]. There is no standard reference gene for all kinds of tissue types. To our best knowledge only few studies investigated the alteration of stability of reference genes in different mesenchymal tumour entities.

In the present study we attempted to identify suitable reference genes in gastrointestinal stromal tumors by using a set of sixteen reference genes which are currently applied in qPCR procedures. Furthermore we evaluated the gene expression of KIT as well as the
alternative RTK FLT3, CSF1-R, PDGFRB, AXL and MET in mutated and non-mutated gastrointestinal stromal tumors by qPCR using the identified reference genes. The study was focussed on wt-GIST compared to samples with mutations in KIT exon 9 and 11 and PDGFRA exon 18 in order to evaluate whether overexpression of these alternative RTK might contribute to the pathogenesis of wt-GIST.

**Methods**

**Samples**

A total of 107 samples were included into this study. All specimens were obtained in the years 2005 and 2006 under approved ethical protocols and with informed consent from each patient. All samples were fixed in neutral-buffered formalin prior to paraffin embedding. 20 samples from normal tissue (i.e., muscularis propria of stomach and gut) as control group and 87 GIST representing different mutational subgroups were evaluated. 20 samples of wt-GIST, 7 samples of wt-GIST associated with neurofibromatosis type 1, 20 samples with exon 9 mutation in KIT, 20 samples with exon 11 mutation in KIT and 20 samples with exon 18 mutation in PDGFRA). Sequence analysis of KIT (exons 8, 9, 11, 13, 14, 15 and 17) and PDGFRA (exons 12, 14 and 18) was carried out as described earlier [1,37,38]. Additionally, in all samples the wild type status of the BRAF gene was ascertained. All GIST samples were stained immunohistochemically for CD117. Four normal tissues from the gastrointestinal region were available as fresh frozen and formalin-fixed, paraffin-embedded samples.

**RNA extraction and cDNA synthesis**

Prior to RNA extraction, paraffin-embedded tissues were cut into 10 μm sections and mounted on glass-slides. Six slides of each specimen were used for RNA extraction. The sections were deparaffinized by extracting twice in xylene for 10 min at room temperature. Rehydration was done in 100% ethanol, 90% ethanol, 80% ethanol and 70% ethanol made with DEPC-water for 10 min each. Tumor areas previously marked on a H&E slide were scraped from the sections with a sterile scalpel. Sections were transferred into a sterile 1.5 ml tube. Extraction and purification of RNA was done using the RNeasy FFPE KIT (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. After tumor localization by H&E staining fresh frozen material was rasped into 10 μm thick pieces and RNA purification was carried out using the RNeasy Kit (Qiagen, Hilden, Germany). Both RNeasy Kits contain a step of DNase treatment. Finally RNA from fresh and from fixed tissue was eluted in water. The quantification was done spectrophotometrically (NanoDrop, PeqLab Technology, Erlangen, Germany). 500 ng of RNA from each sample was reverse-transcribed using a random-hexamer primer and Avian Myoblastosis Virus reverse transcriptase (AMV-RT) according to the manufacturer’s protocol (Qiagen). cDNA from those four fresh frozen and corresponding formalin-fixed, paraffin-embedded (FFPE) control samples was generated in only one reverse transcription and loaded on the reference low density arrays (see below). Further cDNA samples were generated from the four FFPE control tissues, pooled within one patient and loaded onto a second independent reference low density array.

**Identification of reference genes**

The reference genes used here were preselected because of their constitutive, non-regulated stable expression over a wide spectrum of tissues. But nevertheless the preselected reference genes are not suitable for every kind of tissue and therefore need to be analyzed prior to use in a certain study. The detection of suitable reference genes was carried out using TaqMan Low Density Arrays (TLDAs, Microfluidic Cards, Applied Biosystems, Darmstadt, Germany). These arrays are prefabricated 384-well cards where gene-specific primer and probe sets are spotted in small reaction chambers during manufacturing. The cards have 8 separate loading ports leading into 48 wells each. In this study, arrays with triplicates of 16 putative reference genes were used, so 8 different samples could be analyzed. The list of assays is given in table 1. For each sample, 500 ng cDNA was mixed with 2× TaqMan Universal PCR Master Mix (Applied Biosystems). 100 μl of this mixture was loaded into each port and distributed into the reaction chambers by centrifugation. The card was sealed and the quantitative PCR (qPCR) was performed on an ABI PRISM HT 7900 (Applied Biosystems) sequence detection system. After pre-incubation for 2 min at 50°C and 10 min at 95°C, the PCR reaction was performed (15 s at 94°C followed by 60 s at 60°C, 40 cycles). The fluorescent signal was measured in each cycle.

**Analyses of gene expression by qPCR**

qPCR analysis was performed using the assays-on-demand products (Applied Biosystems) listed in table 2. These gene-specific qPCR assays consist of a pair of unlabeled PCR primers and a FAM labeled specific probe. According to the manufacturer of these assays, probe and primer sets that would amplify pseudogenes are excluded in the process of development. Reactions were carried out in a reaction volume containing 5 μl PCR Master Mix (Applied Biosystems), 0.5 μl forward and reverse primer mix, 500 ng cDNA ad 10 μl A.dest. Triplicate reactions were carried out for each transcript. Control reactions were performed using a minus RT preparation and a sample with A.dest instead of RNA.
PCR conditions were the same as for the TaqMan Low Density Arrays.

Statistical analyses
The geNorm applet for Microsoft Excel was used to determine the most stable genes among the sixteen candidate reference genes. Raw Cq values were converted into relative quantities for analysis with geNorm, where the highest relative quantity for each gene is set to 1. The program selects from a panel of candidate reference genes the two most stable genes or a combination of multiple stable genes for normalization. The gene expression stability (M) value is based on the combined estimate of intra- and intergroup expression variations of the genes studied and takes the PCR efficiency into account. The limited M-value is 1.5. The most stable genes are stepwise selected from the investigated gene panel to estimate how many reference genes should be used. The normalization factors define the optimal number of reference genes required for a precise normalization design. The analysis of the expression data of tumor samples compared to control samples was performed with the REST software (Relative Expression Software Tool) [39]. The software normalizes the measured Cq-values of the target genes with those of the reference genes and compares the expression data of tumor and control samples by considering the PCR efficiency and the mean crossing point deviation.

Results
RNA quality
RNA quality of the samples was inspected on a 1% agarose gel. The concentration and purity of the RNA was characterized by the mean A260/280 ratio and was on average 1.99 for fresh frozen as well as for formalin fixed, paraffin-embedded samples and reflected pure and protein-free RNA.

Expression variability of cDNA synthesis
Four normal control FFPE tissue samples from the gastric and bowel wall were used to determine suitable reference genes. One TLDA was done with cDNA from only one reverse transcription for each sample. As it is assumed that cDNA synthesis from FFPE tissue varies strongly in efficiency, pooled cDNA synthesis samples from the same FFPE tissue were used for a second TLDA. The pooled cDNA preparation showed an essentially lower variance in contrast to the cDNA samples from only one reverse transcription (Figure 1). The median of the M-values ranges

| Ch | Function | Assay-on-demand |
|----|----------|-----------------|
| 5  | tyrosine protein kinase receptor | Hs00234617_m1 |
| 13 | tyrosine protein kinase receptor | Hs00174690_m1 |
| 4  | tyrosine protein kinase receptor | Hs00174029_m1 |
| 5  | tyrosine protein kinase receptor | Hs00387362_m1 |
| 7  | hepatocyte growth factor receptor | Hs00179845_m1 |
| 19 | tyrosine protein kinase receptor | Hs00242357_m1 |
from 0.1 to 0.2, thus the variability of the technical replicates is low. Within a single specimen the range is also very small. The median of the cDNA samples from a single reverse transcription ranges from 0.1 to 0.4. However, each cDNA sample from one reverse non-pooled transcription showed a high variance of the values (median of M-values 0.1 to 0.8, Figure 1). Therefore the variance of the samples can be lowered by cDNA pooling. The median of the cDNA samples from one reverse transcription from fresh frozen tissue ranged from 0.1 to 0.2 and can be compared to the median of the pooled cDNA from the FFPE tissue. Within a single specimen the range is higher than in the pooled cDNA samples, but smaller than in the cDNA from only one reverse transcription from the FFPE samples.

**Identification of suitable reference genes for normalization**

All 16 putative reference genes had a high expression stability and the ‘M’-value (0.02-0.06) was clearly below the ‘M’-cutoff-value of 1.5. By stepwise exclusion of genes, the expression stability value ‘M’ is calculated (data not shown) and the expression stability rises with the exclusion of further genes. Samples generated from only one reverse transcription, showed POLR2A, TFRC, RPLPO and GAPDH as the most stable genes (Figure 2a). The average stability value was between 0.05 and 0.07. The range of the values was 0.01-0.15. The most stable genes for pooled cDNA samples were PGK1, PPIA, RPLPO and IPO8 with a median of 0.04-0.05 (Figure 2b). The range of the values was lower and varied between 0.01 and 0.09. All in all the gene expression variability of the pooled cDNA samples is much lower compared to the single reverse transcription cDNA synthesis. The gene UBC is the most stable one in fresh frozen tissue with the smallest value range (0.01-0.08), but RPLPO and PPIA belong also to the most stable genes in fresh frozen tissue with a median of 0.03-0.045 and the range of the values was 0.1-0.12 (Figure 2c). TBP was not expressed in the tissue. By combining the lowest variability values of fixed and fresh tissue, the genes POLR2A, PPIA, RPLPO and TFRC were detected (Figure 3). Hence these four genes were used for further analysis with the GIST samples.
Expression profiles of KIT, FLT3, CSF1-R, PDGFRB, AXL and MET

Six patient groups (NT = normal tissue -control, WT = wild type, Ex9 = KIT Exon 9 mutated, Ex11 = KIT Exon 11 mutated, NF1 = wild type and neurofibromatosis type 1, Ex18 = PDGFRA Exon 18 mutated) were used to analyze the gene expression of KIT, FLT3, CSF1R, PDGFRB, AXL and MET. Here, the REST analysis with POLR2A is shown exemplarily. The REST analysis of the target genes KIT, CSF1R, FLT3, PDGFRB, AXL and MET with the reference genes RPLPO, PPIA and TRFC showed the same significant results for the differential expression or at least the same trend in mutated and non-mutated GIST (data not shown). As suspected, we found a significant overexpression of KIT in exon 9 and exon 11 mutated GIST in comparison with normal tissue. Also a significantly lowered expression of PDGFRB in both groups compared to normal tissue was shown. The same effect was observed in PDGFRA exon 18 mutated GIST compared to normal tissue (table 3).

On closer inspection of normal tissue and wt-GIST a clear overexpression of KIT in the tissue of wild type tumors was shown. The same effect, but even stronger, could be detected by comparing NF1-associated wt-GIST with normal tissue. Concerning KIT expression, the tumors without mutation behave like KIT mutated GIST (table 3). This was in accordance with immunohistochemical staining (Figure 4).

The other target genes, FLT3, CSF1R, PDGFRB, AXL and MET showed lower expression levels in the tumor tissue compared to normal tissue except AXL in NF1 associated GIST. MET was even significantly reduced in

**Figure 2** Expression variability of reference genes. Analysis of reference gene expression on TaqMan Low Density Arrays. a) cDNA from a single RT-transcription. b) pooled cDNA samples from normal tissue. The genes POLR2A, TFRC, PPIA and RPLPO had the lowest expression variability. c) cDNA from fresh frozen tissue. RPLPO, PPIA and UBC had the lowest expression variability, TBP was not expressed in the tissue. On the x-axis, the stability values are plotted. Whiskers represent range of data of 4 samples.
wt-GIST, KIT exon 9, 11 and PDGFRA exon 18 mutated GIST. No changes in expression levels were detected comparing normal tissue and wt-GIST with KIT exon 11 mutated GIST, indicating that both groups possess similar expression levels for the analyzed receptors. None of the groups showed significant expression alterations for CSF1R and FLT3. An exception is the group of KIT exon 9 mutated GIST. A lower expression of CSF1R, FLT3, PDGFRB and MET compared to normal tissue was identified in contrast to KIT, which showed a significant overexpression. In summary, overexpression of KIT was detected in each GIST subgroup compared to the corresponding normal tissue by using the preassigned reference gene POLR2A (table 3). GIST with a mutation in exon 18 of PDGFRA did not show a significant upregulation of KIT compared to normal tissue. GIST with a mutation in KIT exon 9, 11 and in the PDGFRA exon 18 showed significant PDGFRB downregulation.

Discussion
Most gastrointestinal stromal tumors exhibit mutations in exon 11 of the KIT gene. This exon encodes for the juxtamembrane domain of the receptor which possesses an autoinhibitory function. In wt-GIST, activating mutations are found neither in the KIT nor in the PDGFRA gene. Clinical treatment with the tyrosine kinase inhibitor imatinib targets the ATP binding site in the kinase domain of the KIT receptor. In wt-GIST, response to

Table 3 Expression analysis of target genes

| Gene | Factor | p-value | Gene | Factor | p-value |
|------|--------|---------|------|--------|---------|
| KIT  | 5.9+   | 0.095   | KIT  | 17+    | 0.003   |
| CSF1R| 3.99-  | 0.217   | CSF1R| 2.99-  | 0.294   |
| FLT3 | 2.43-  | 0.498   | FLT3 | 3.985- | 0.179   |
| PDGFRB| 5.79- | 0.087   | PDGFRB| 2.827-| 0.196   |
| AXL  | 2.1-   | 0.66    | AXL  | 2.5+   | 0.53    |
| MET  | 8.7-   | 0.034   | MET  | 6.5-   | 0.13    |

Expression analysis of KIT, CSF1R, FLT3, PDGFRB, AXL and MET was carried out in six GIST cohorts (NT = normal tissue, WT = wild type, Exon 9, Exon 11, NF1 = neurofibromatosis type 1, Exon 18). Significant variances are in bold. The expression data were calculated using the REST software with POLR2A as reference gene. NT: normal tissue; p < 0.05; +/- indicates up- or downregulation.

Figure 3 GeNorm analysis of selected reference genes. Calculation of the average expression stability of the remaining 4 candidate reference genes for normalization in GIST by GeNorm analysis. The least stable gene with the highest 'M'-value is indicated from left to right on the x-axis. After combining the data of the single RT-transcription cDNA and the pooled cDNA the stepwise exclusion of the least stable reference genes resulted in POLR2A and TRFC.
treatment is often poor [12] although most of them overexpress the KIT protein. A screen for activating mutations in the juxtamembrane domain of the alternative receptor tyrosine kinases CSF1R, FLT3, PDGFRB in 30 wt-GIST samples yielded only wild type sequences (data not shown) in agreement with previously published results [40]. Therefore we investigated in this study whether the expression of alternative receptor tyrosine kinases may contribute to the pathogenesis of wt-GIST and thus may help to identify wt-GIST subgroups with different response to imatinib and elucidate novel therapeutic targets. The pathogenesis of several malignant tumors is associated with overexpression of CSF1R and PDGFRB [24,28]. Imatinib inhibits cell invasion in malignant peripheral nerve sheath tumors by blocking PDGFRB [41] and it has been found to have antitumor activity in patients with chordoma [42]. In our study, the gene-expression level of CSF1R, FLT3 and PDGFRB was determined in a cohort of 87 GIST samples. Furthermore, we assessed the expression of AXL and MET, two receptor tyrosine kinases which were found to be alternatively activated in therapy resistant GIST [19].

Gene expression analysis by qPCR requires suitable reference genes. The expression of reference genes like GAPDH or BETA-ACTIN is regulated differentially depending on the tissue type. Therefore they are not suited as univocal reference genes [35,43]. The determination of reference genes with stable expression in the experimental system used is essential to ensure accurate normalization and interpretation of results.

Whereas fresh tissue is frequently not available for genetic analysis, FFPE material is the standard. After formalin-fixation and paraffin-embedding of tissue, the isolated RNA is often heavily fragmented. In our study we used fresh as well as fixed material from the gastrointestinal tract to determine suitable reference genes and to analyze whether their expression levels are comparable. We then validated the reference genes by qPCR in our GIST cohort.

As reported also by others [44,45] the variability of expression levels for the reference genes was very diverse comparing fresh frozen and formalin-fixed tissue. Therefore, we decided to validate separate reference genes for each tissue type. The genes TRFC, POLR2A, PPIA and RPLPO were validated as appropriate reference genes for FFPE tissue. For fresh frozen tissue, PPIA and RPLPO were also found to be suitable. Additionally, UBC is suited as a reference gene in fresh frozen tissue. The variability of the reference genes for fresh frozen tissue was lower than for fixed tissue. To overcome the problem of high variability in FFPE tissue, we pooled two independent cDNA syntheses from one sample as recommended in the MIQE guidelines [30,46]. The MIQE guidelines give considerations for a consistent application of the qPCR technology including experimental details, data analysis and reporting principles. Unequal efficiency of cDNA synthesis might be a reason for deviation. Additionally, we tried to select consistent patient material for our cohort by choosing paraffin blocks having the same age. It was shown by Bibikova [45], that Cq-values in qPCR experiments depend on the age and condition of the tissue blocks. Because the expression value depends also on amplicon length [30,44], only primer sets generating amplicons of about 100 bp were chosen for qPCR. This corresponds to the fragment length of degraded RNA between 100 and 200 bp. The application of the MIQE...
guidelines results in a minimum variability for reference genes. In summary, considering the MIQE guidelines FFPE material can be used reliably for expression analysis in GIST, but the use of separate reference genes for FFPE tissue is indispensable.

Our qPCR analysis included GIST with wild type sequences in the hot-spot regions of KIT and PDGFRA (wt-GIST), KIT exon 9 and exon 11 mutated samples, PDGFRA exon 18 mutated samples, NF-1-associated wt-GIST and normal tissue controls. The wt-GIST and the samples with the two different KIT mutations showed a significantly increased expression of KIT in contrast to the normal tissue. The results correspond to the immunohistochemical stainings of our samples and were in agreement with published data [47]. It was shown immunohistochemically that PDGFRA mutated GIST have only a slightly increased protein expression of KIT [13]. Our data revealed the same trend on RNA level compared to the normal tissue. Additionally, we could show in all groups of mutated GIST a significantly reduced expression of PDGFRB compared to the normal tissue. This could be due to the concomitant increase of KIT expression. When comparing NF-1-associated GIST without KIT mutation with wt-GIST without NF-1 association the latter showed a lower expression of KIT. Thus, the results of the two wt-GIST groups give a heterogeneous profile, which suggests that different genomic events may be responsible for the development of these tumors.

Comparing our sample groups with each other, no significant difference in the gene expression levels of FLT3, CSFIR and AXL were determined. An exception was seen in the sample group with KIT exon 9 mutation. Here, a significantly reduced expression of CSFIR, FLT3 and PDGFRB compared to the normal tissue was detected. The results lead us to the assumption that KIT exon 9 mutated GIST play a special role compared to GIST carrying other mutations. Interestingly, KIT exon 9 mutated GIST need a double daily dose of the tyrosine kinase inhibitor imatinib to be effectively treated [48]. Furthermore, they develop preferentially in the small intestine but only rarely in the stomach where the majority of GIST are detected [49].

All qPCR data were calculated four times with TRFC, POLR2A, PPIA and RPLPO as reference genes using the REST software. Concordant results with the four reference genes are based on the extensive and complex preselection of our cohort and the preparation of cDNA synthesis according to the MIQE guidelines.

Conclusions
In summary, we conclude that none of the alternative receptor tyrosine kinases analyzed here are associated with the pathogenesis of wild type or mutated GIST. It remains to be clarified whether an increased expression of receptor tyrosine kinase ligands is responsible for tumorigenesis of wt-GIST as it is described for dermatoﬁbrosarcoma protuberans (DFSP) and tenosynovial giant cell tumor (TGCT) [25,50]. Further studies are needed to elucidate the role of ligand-driven pathogenesis in wt-GIST.

Acknowledgements and Funding
This study was supported by a grant of the BONFOR programme of the Bonn Medical centre to HUS.

Author details
1Department of Pathology, University of Bonn Medical Center, Bonn, Germany. 2Division of Molecular Genetics, DKFZ, Heidelberg, Germany. 3Department of Integrative Bioinformatics and Systems Biology, DKFZ, Heidelberg, Germany. 4Department of Hematology and Oncology, Otto-von-Guericke-University Magdeburg, Germany.

Authors’ contributions
MICB, JF and HK carried out the molecular genetic studies and drafted the manuscript. SMB and HUS developed the design of the study. SMB coordinated the study together with EW and HUS. BB and MZ performed the statistical analysis of the expression data. EW and HUS participated in the diagnosis and selection of tumor material. RB revised the manuscript for important intellectual content and approved the final manuscript. All authors read and approved the manuscript.

Received: 30 July 2010 Accepted: 20 December 2010
Published: 20 December 2010

References
1. Pauls K, Merkelbach-Bruse S, Thal D, Buttrner R, Wardellmann E. PDGFRalpha- and c-kit-mutated gastrointestinal stromal tumours (GISTs) are characterized by distinctive histological and immunohistochemical features. Histopathology 2005, 46:166-175.
2. Sarlomo-Rikala M, Kovatch AJ, Barusviucius A, Miettinen M. CD117: a sensitive marker for gastrointestinal stromal tumors that is more specific than CD34. Mod Pathol 1998, 11:728-734.
3. Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Hale P, Town A, et al. PDGFRα activating mutations in gastrointestinal stromal tumors. Science 2003, 299:708-710.
4. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 1998, 279:557-560.
5. Lasota J, Miettinen M. Clinical significance of oncogenic KIT and PDGFRA mutations in gastrointestinal stromal tumours. Histopathology 2008, 53:245-266.
6. Rubin BP, Singer S, Tsoa C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen CJ, Xiao S, Tuveson DA, et al. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res 2001, 61:8118-8121.
7. Wardellmann E, Losen I, Hans V, Neidt I, Spiedel N, Bierhoff E, Heinicke T, Fietzsch T, Buttrner R, Merkelbach-Bruse S. Deletion of Trp-557 and Lys-558 in the juxtamembrane domain of the c-kit protooncoprotein is associated with metastatic behavior of gastrointestinal stromal tumors. Int J Cancer 2003, 106:887-895.
8. Lasota J, Miettinen M. KIT and PDGFRA mutations in gastrointestinal stromal tumours (GISTs). Semin Diagn Pathol 2006, 23:91-102.
9. Medeiros F, Corless CL, Duensing A, Hermick JL, Oliveira AM, Heinrich MC, Fletcher JA, Fletcher CD. KIT-negative gastrointestinal stromal tumors: proof of concept and therapeutic implications. Am J Surg Pathol 2004, 28:889-894.
10. Miettinen M, Fietzsch JF, Sobin LH, Lasota J. Gastrointestinal stromal tumors in patients with neurofibromatosis 1: a clinicopathological and molecular genetic study of 45 cases. Am J Surg Pathol 2006, 30:90-96.
11. Miettinen M, Lasota J, Sobin LH. Gastrointestinal stromal tumors of the stomach in children and young adults: a clinicopathologic,
immunohistochemical, and molecular genetic study of 44 cases with long-term follow-up and review of the literature. Am J Surg Pathol 2005, 29:1373-1381.

12. Debrecy-Rychter M, Dumetz H, Jodoon I, Wasan B, Verweij J, Brown M, Dimitrijevic S, Scott R, Stul M, Vranck H et al. Use of c-KIT/PGDFR mutational analysis to predict the clinical response to imatinib in patients with advanced gastrointestinal stromal tumours entered on phase I and II studies of the EORTC. Soft Tissue and Bone Sarcoma Group. Eur J Cancer 2004, 40:689-695.

13. Hostein I, Faur N, Primois C, Bouy F, Denard J, Emile JF, Birnbaum D, Rosnet O, Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, Scherer SW, Tamborini E, Miselli F, Negri T, Lagongino MS, Staurengo S, Dagrada GP, Scaglotti S, Pastore E, Perino F, et al. Molecular and biochemical analyses of platelet-derived growth factor receptor (PDGFR) B, PDGFR, and KIT receptors in chordomas. Clin Cancer Res 2006, 12:6920-6928.

14. Bustin SA, Benes V, Gannon JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009, 55:611-622.

15. Feige S, Pfaffl MW: RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 2006, 27:126-139.

16. Ohl F, Jung M, Radonic A, Sachs M, Loening SA, Jung K: Identification and validation of suitable endogenous reference genes for gene expression studies of human bladder cancer. J Urol 2006, 175:1915-1920.

17. Saviozzi S, Cordero F, Lo Iacono M, Novello S, Scaglotti GV, Calogero RA: Selection of suitable reference genes for accurate normalization of gene expression profile studies in non-small cell lung cancer. BMC Cancer 2006, 6:200.

18. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002, 3:RESEARCH0034.

19. Bustin SA, Benes V, Nolan T, Pfaffl MW: Quantitative real-time RT-PCR-a perspective. J Mol Endocrinol 2005, 34:597-608.

20. Bustin SA, Nolan T, Pfaffl MW: Quantitative real-time reverse transcription polymerase chain reaction: normalization to RNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 2002, 309:293-300.

21. Vandewalle E, Neidt I, Berhof E, Speidel N, Manegold C, Fischer HP, Pfeiffer U, Pietsch T: c-kit mutations in gastrointestinal stromal tumours occur preferentially in the spindle rather than in the epithelioid cell variant. Mod Pathol 2002, 15:125-136.

22. Vandewalle E, Thomas N, Merkelbach-Bruse S, Pauls K, Speidel N, Buttner R, Bihl H, Leuteneg T, Hohenberger P: Acquired resistance to imatinib in gastrointestinal stromal tumours caused by multiple KIT mutations. Lancet Oncol 2005, 6:249-251.

23. Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002, 30:e36.

24. Sihto H, Franssila K, Tanner M, Yasama-Nolvi C, Sarlomo-Rikala M, Nupponen NN, Joensuu H, Isola J: Platelet-derived growth factor receptor family mutations in gastrointestinal stromal tumours. Scand J Gastroenterol 2006, 41:805-811.

25. Aoki M, Nabeshima K, Koga K, Hamasaki M, Suzumiya J, Tamura K, Iwasaki H: Imatinib mesylate inhibits cell invasion of malignant peripheral nerve sheath tumour induced by platelet-derived growth factor-BB. Lab Invest 2007, 87:767-779.

26. Casali PG, Mersina A, Scattoni S, Tamborini E, Crippa F, Gronchi A, Orlandi R, Ripamonti C, Spreafico F, Bertoni R, et al: Imatinib mesylate in chordomas. Cancer 2004, 101:2086-2097.

27. Bustin SA: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000, 25:199-193.

28. Antonov J, Goldstein DR, Oberli A, Baltzer A, Pirotta M, Fleischmann A, Reichardt P: Why the need for qPCR publication guidelines-The case for MIQE. Methods 2010, 50:217-226.

29. Miettinnen M, Sobin LH, Sarlomo-Rikala M: Immunohistochemical spectrum of GSTs at different sites and their differential diagnosis with a reference to CD117 (KIT). Mod Pathol 2000, 13:114-119.

30. Reichardt P: Optimal use of targeted agents for advanced gastrointestinal stromal tumours. Oncology 2010, 78:130-140.
49. Emory TS, Sobin LH, Lukes L, Lee DH, O’Leary TJ. Prognosis of gastrointestinal smooth-muscle (stromal) tumors: dependence on anatomic site. Am J Surg Pathol 1999, 23:82-87.

50. Simon MP, Pedeutour F, Sirvent N, Grosgeorge J, Minoletti F, Coindre JM, Terrier-Lacombe MJ, Mandahl N, Craver RD, Bin N, et al. Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. Nat Genet 1997, 15:95-98.

doi:10.1186/1471-2199-11-100
Cite this article as: Fassunke et al. qPCR in gastrointestinal stromal tumors: Evaluation of reference genes and expression analysis of KIT and the alternative receptor tyrosine kinases FLT3, CSF1-R, PDGFRB, MET and AXL. BMC Molecular Biology 2010 11:100.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit