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microRNA expression signatures of gastrointestinal stromal tumours: associations with imatinib resistance and patient outcome

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Background: Gastrointestinal stromal tumour (GIST) is mainly initialised by receptor tyrosine kinase gene mutations. Although the tyrosine kinase inhibitor imatinib mesylate considerably improved the outcome of patients, imatinib resistance still remains a major therapeutic challenge in GIST therapy. Herein we evaluated the clinical impact of microRNAs in imatinib-treated GISTs.

Methods: The expression levels of microRNAs were quantified using microarray and RT–qPCR in GIST specimens from patients treated with neoadjuvant imatinib. The functional roles of *miR-125a-5p* and PTPN18 were evaluated in GIST cells. PTPN18 expression was quantified by western blotting in GIST samples.

Results: We showed that overexpression levels of *miR-125a-5p* and *miR-107* were associated with imatinib resistance in GIST specimens. Functionally, *miR-125a-5p* expression modulated imatinib sensitivity in GIST882 cells with a homozygous *KIT* mutation but not in GIST48 cells with double *KIT* mutations. Overexpression of *miR-125a-5p* suppressed PTPN18 expression, and silencing of PTPN18 expression increased cell viability in GIST882 cells upon imatinib treatment. PTPN18 protein levels were significantly lower in the imatinib-resistant GISTs and inversely correlated with *miR-125a-5p*. Furthermore, several microRNAs were significantly associated with metastasis, *KIT* mutational status and survival.

Conclusions: Our findings highlight a novel functional role of *miR-125a-5p* on imatinib response through PTPN18 regulation in GIST.

Gastrointestinal stromal tumour (GIST) is the most common mesenchymal tumour of the gastrointestinal tract with an incidence of 1–2 cases/100 000 inhabitants per year (Nilsson *et al*, 2005). The main initial event in GIST tumorigenesis is gain-of-function mutations of the receptor tyrosine kinase genes *KIT* or *PDGFRA*. These mutations occur in approximately 85% of GISTs (Corless *et al*, 2004) and cause ligand-independent activation of the encoded receptor tyrosine kinases that results in aberrant and uncontrolled cell growth and tumour formation (Corless *et al*, 2004). The development of imatinib mesylate, a small-molecule inhibitor that targets several receptor tyrosine kinases, including KIT and PDGFRA, markedly improved patient outcome. However, the efficacy of imatinib treatment varies depending on the mutated domains of *KIT* and *PDGFRA* (Gramza *et al*, 2009). Still, 10% of GIST patients have primary resistance to imatinib, and 40–50% of initially responding

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patients develop secondary resistance and progress within 2 years (Gramza *et al*, 2009).

Previous studies have suggested that acquisition of second site mutations in the kinase or loop domain of *KIT* or *PDGFRA* is the main mechanism of resistance to imatinib therapy in GIST (Gramza *et al*, 2009). However, second site mutations are not detected in a substantial proportion of imatinib-resistant GISTs (Antonescu *et al*, 2005; Wardelmann *et al*, 2006), suggesting the involvement of additional mechanisms. Furthermore, the mechanism of primary resistance remains unclear. Therefore, a better understanding of the underlying mechanisms in imatinib resistance is needed for improved treatment planning and development of additional treatment strategies.

microRNAs (miRNA) are short RNAs of ~22 nucleotides in length that regulate many physiological and pathological processes, by forming imperfect hybrids with the 3′ untranslated region sequences of their target mRNAs that leads to mRNA degradation and/or translational repression (Bartel, 2004). Distinct miRNA expression signatures have been found in a variety of cancers, and they are often associated with patient outcome, suggesting their potential use as diagnostic and prognostic markers. Recent studies have shown their importance in drug resistance of different cancer types (Zheng *et al*, 2010). In GIST, specific miRNA expression signatures are associated with chromosome 14q loss (Choi *et al*, 2010; Haller *et al*, 2010), anatomical site (Choi *et al*, 2010; Niinuma *et al*, 2012), *KIT* or *PDGFRA* mutation (Haller *et al*, 2010), tumour risk (Choi *et al*, 2010; Niinuma *et al*, 2012) and overall survival (Niinuma *et al*, 2012). However, no study has addressed the functional role of miRNAs in imatinib response in GISTs, and it is unclear whether miRNAs could be used as prognostic markers for GIST patients who received neoadjuvant imatinib therapy.

In this study, we evaluated the clinical impact of miRNA deregulation in relation to imatinib response, clinical features and *KIT* mutational status of GISTs. We further investigated the functional role of *miR-125a-5p* and its potential targets in imatinib resistance of GIST cells and assessed the target protein expression levels in GIST clinical specimens.

MATERIALS AND METHODS

Clinical samples. A total of 30 snap-frozen tumours from 24 GIST patients who had received neoadjuvant imatinib treatment were included in this study. All tissue samples were collected at the Karolinska University Hospital Biobank between 2003 and 2012. All samples were obtained with informed consent, and study of the tissue materials was approved by the Karolinska Institutet ethical committee at Karolinska Hospital, Stockholm, Sweden. The patients were followed up until September 2012 or the time of death. The clinical, histopathological and follow-up details of all cases are detailed in Table 1, and a subset of the cases were included in a previous study (Ahlen *et al*, 2012). The diagnosis of GIST was based on the routine histopathological examination and positive immunoreactivity for CD117 (KIT). Clinical imatinib response was evaluated by fluorodeoxyglucose positron emission tomography and/or contrast-enhanced computed tomography for (i) tumour size, (ii) tumour characteristics, such as tumour density, glucose uptake, and intra-tumour alterations (e.g., addition of denser subparts of the tumour) and (iii) peritoneal and/or liver metastasis. Tumours that continued to progress under imatinib treatment were classified as imatinib resistant, while the tumours that shrunk obviously or showed significant cystic changes or necrosis were grouped as imatinib sensitive.

GIST cell lines. Two GIST cell lines (GIST882 and GIST48) were kindly provided by Dr Jonathan Fletcher at Brigham and Women's Hospital, Boston, MA, USA. The GIST882 cell line was established from an untreated GIST with a homozygous missense mutation in *KIT* exon 13 (K642E), and it is imatinib sensitive (Tuveson *et al*, 2001). GIST882 cells were grown in RPMI 1640 media supplemented with 15% fetal bovine serum, 2 mM L-glutamine and 1% penicillin–streptomycin. The GIST48 cell line was established from a progressed GIST with a primary homozygous *KIT* exon 11 missense mutation (V560D) and a heterozygous *KIT* secondary exon 17 (D820A) mutation, and it is imatinib resistant (Bauer *et al*, 2006). GIST48 cells were grown in F-10 media supplemented with 15% fetal bovine serum, 2.5 $\mu g\,ml^{-1}$ MITO + serum extender (BD Biosciences, Franklin Lakes, NJ, USA), 5 $\mu g\,ml^{-1}$ bovine pituitary extract (BD Biosciences), 2 mM L-glutamine and 1% penicillin–streptomycin. Both cell lines were cultured at 37 °C with 5% CO_2. The authentication of the cell lines was confirmed by *KIT* mutation analysis and CD117 immunoreactivity (Berglund *et al*, 2013). The possibility of cross-contamination by other cell lines was ruled out by short tandem repeat (STR) profiling performed by Bio-Synthesis Inc. (Lewisville, TX, USA). The STR profiles of both cell lines are shown in Supplementary Table S1.

DNA and RNA extraction. Genomic DNA was purified using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), and total RNA isolation was performed using the mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA). DNA and RNA concentrations were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

***KIT* and *PDGFRA* mutation analysis.** All GIST samples were analysed for *KIT* (exons 9, 11, 13 and 17) and *PDGFRA* (exons 12 and 18) mutations using a direct sequencing method. Mutation analyses of 12 samples had been performed in the routine pathology diagnostic laboratory at Karolinska University Hospital, while 18 samples were analysed in this study. Primer sequences for amplification of genomic DNA were previously described (Sihto *et al*, 2005) and are available in Supplementary Table S2. PCR amplifications were performed in 25 μl reactions containing 1 unit Platinum *Pfx* DNA Polymerase (Life Technologies), 1 × PCR buffer, 0.2 mM dNTPs, 2 mM $MgSO_4$, 0.2 μM forward and reverse primers and 250 ng genomic DNA. A touch-down protocol was used with the following conditions: 2 min at 94 °C; 10 cycles of (30 s at 94 °C, 45 s at 66 °C–56 °C (by decreasing the annealing temperature 1 °C per cycle) and 30 s at 68 °C); 35 cycles of (30 s at 94 °C, 1 min at 56 °C and 30 s at 68 °C); followed by extension for 7 min at 68 °C. The PCR products were cleaned up using the ExoSAP-IT (USB Corporation/Affymetrix, Santa Clara, CA, USA) and sequenced at the KIGene facility (Stockholm, Sweden).

Genome-wide miRNA expression profiling. Global miRNA expression profiling was performed in 17 GISTs using the Human Agilent's miRNA microarray system (Agilent, Santa Clara, CA, USA) with probes matching 903 human miRNAs (miRBase release 14). Array hybridisations and data analyses were performed as previously described (Caramuta *et al*, 2010). Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used for normalisation and median centering. Normalised miRNAs with <20% missing values were included for Significance Analysis of Microarrays (SAM) (http://www-stat.stanford.edu/Btibs/SAM/) and clustering analysis. Visualisation of the clustering data was done using Java TreeView (http://jtreeview.sourceforge.net). The data from microarray analysis have been deposited at NCBI Gene Expression Omnibus (GEO accession number, GSE45901).

TaqMan reverse transcription quantitative PCR (RT–qPCR) analysis. The expression of 10 selected mature miRNAs, i.e., *miR-301a-3p*, *miR-30c*, *miR-134*, *miR-1915*, *miR-125a-5p*, *miR-365*, *miR-107*, *miR-150-3p*, *miR-1207-5p* and *miR-638*, as well as *PTPN18* and *STARD13* mRNAs, was quantified using commercially available TaqMan RT–qPCR assays (Life Technologies).

Table 1. Details of mutations, clinical features and imatinib treatment for the 24 GIST patients

		Mutations detected		Tumour histopathology			GIST at the start of IM		IM treatment				Follow-up	
Case no.	Gender/ age (years)	*KIT*	*PDGFRA*	Size	MI	MIB-1	Type	Site	Preop	Postop	IM response At op	Metastasis	Time	Outcome
GIST1	M/39	p.K558 V559>N and p.R804W	wt	5	40	40	Primary	Gastric	36 m	Yes	Resistant	Liver	48	DOD
GIST2	F/39	wt	p.I843 D846del	2	>10	20	Recurrent	Jejunum	15 m	Yes	Resistant	Liver	24	DOD
GIST3	M/58	p.W557R	wt	13	0–2	1	Primary	Duodenum	10 m	Yes	Resistant	Liver	108	DOD
GIST4	M/52	p.E554 K558del	wt	23	3–9	10	Primary	Rectum	>5 m	Yes	Resistant	Liver	27	DOD
GIST5	M/58	wt	p.D842V	16	2	1	Primary	Gastric	3 m	Yes	Resistant	Liver	24	Dead
GIST6	M/72	p.W557R	wt	>10	33	30	Recurrent	Gastric	>14 m	Yes	Resistant	Liver	50	DOD
GIST7	F/59	p.V560D and p.D820Y	wt	3.7	NA	10	Recurrent	Rectum	>96 m	Yes	Resistant	Liver	160	Alive
GIST8	M/33	p.W557R	wt	10	>10	20	Recurrent	Jejunum	18 m	Yes	Resistant	No	30	Alive
GIST9[a]	F/44	p.K550 P551>I and p.D820G	wt	4.5–30	>100	NA	Recurrent	Jejunum	10 m	Yes	Resistant	Peritoneum	28	DOD
GIST10[a]	M/46	p.V559D and p.V654A	wt	1–9.3	>10	NA	Primary	Jejunum	>48 m	Yes	Resistant	Peritoneum	64	Alive
GIST11	F/72	wt	wt	14	>100	5	Recurrent	Jejunum	12 m	Yes	Sensitive	Peritoneum	109	Alive
GIST12	F/59	p.W557 V559>C	wt	7	23	20	Primary	Gastric	5 m	No	Sensitive	Liver	82	Alive
GIST13	F/54	p.L576 P577insQL	wt	12	0	1	Primary	Gastric	12 m	Yes	Sensitive	No	98	Alive
GIST14	M/35	p.W557 N566del	wt	3	0–2	1	Primary	Rectum	10 m	Yes	Sensitive	No	46	Alive
GIST15	F/51	p.P585P	wt	6	>10	10	Primary	Rectum	18 m	No	Sensitive	No	66	Alive
GIST16	M/62	p.W557R	wt	5	1	NA	Primary	Jejunum	8 m	No	Sensitive	No	21	Dead
GIST17	F/62	p.V559D	wt	9	0–2	30	Primary	Gastric	9 m	Yes	Sensitive	Liver	44	Alive
GIST18	F/71	p.L576P	wt	6	0	NA	Primary	Rectum	6 m	Yes	Sensitive	No	27	Alive
GIST19	M/37	p.V559D	wt	15	5	10	Primary	Rectum	10 m	Yes	Sensitive	No	47	Alive
GIST20[b]	M/44	p.W557 V559>C and p.V654A	wt	11	NA	30	Primary	Gastric	8 m	Yes	Sensitive	Liver	38	DOD
GIST21	M/50	p.V560D	wt	5	8	5	Primary	Rectum	12 m	Yes	Sensitive	No	62	Alive
GIST22	M/48	p.V559 N566>D	wt	3	2	1	Primary	Gastric	4 m	Yes	Sensitive	No	27	Alive
GIST23	M/24	wt	wt	10	0	NA	Primary	Gastric	2 m	Yes	Sensitive	No	25	Alive
GIST24	M/67	wt	p.I843 D846del	17	68	25	Primary	Duodenum	6 m	Yes	Sensitive	Liver	60	DOD

Abbreviations: DOD = dead of disease; F = female; IM = matinib; m = months; M = male; MI = Mitotic index, i.e., the number of mitotic figures per 50 high power fields (HPF); MIB-1 = proliferation index with % of Ki-67-positive nuclei; NA = not available; op = operation; postop = postoperative imatinib treatment; preop = preoperative duration of imatinib treatment; wt = wild-type.

[a]GIST9 and GIST10 had four tumours, three of them were non-responding and one was responding (i.e., GIST9-3 and GIST10-3).

[b]Histologically, this tumour had two areas, one area showed highly necrosis and the other area had many viable tumour cells and mitotic cells.

cDNAs were synthesised from 40 ng total RNA using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies), or from 100 ng total RNA using High Capacity cDNA Reverse Transcriptase Kit (Life Technologies). RT–qPCR was performed using the TaqMan Universal PCR Master Mix (Life Technologies) in a 7900HT Real-time PCR System (Life Technologies). *RNU6B* and *18S* rRNA was used as endogenous controls for miRNAs and mRNAs, respectively. All reactions were performed in triplicate, and relative expression levels were determined with the ΔC_T method and reported as $2^{-\Delta CT}$.

miRNA transfection in GIST882 and GIST48 cells. GIST882 and GIST48 cells were transfected using Nucleofector technology (Lonza AG, Basel, Switzerland). In brief, 4×10^6 cells were resuspended in 100 μl Nucleofector solution V and mixed with 100 pmol of miRNA mimic (pre-miR-125a-5p, pre-miR-211 or pre-miR-944), pre-miR Negative control (pre-miR-CTR), miRNA inhibitor (anti-miR-125a-5p) or anti-miR Negative control (anti-miR-CTR) (Life Technologies). Cells were then electroporated using the program T-20, seeded in T-25 flasks with 4 ml media and cultured at 37 °C for 48 h before imatinib cytotoxicity assay. Transfection efficiency was determined by measuring the cellular *miR-125a-5p* levels using RT–qPCR (Supplementary Figure S1A).

Transfection of short hairpin RNAs (shRNAs). shRNA against human *PTPN18* (target sequence: 5′-GAGGGACACAGCGAC

TACA-3′; Gensler *et al*, 2004) was cloned into pcDNA3-U6M2 vector as described previously (Taft *et al*, 2011). Primers are included in Supplementary Table S2. A total of 3×10^6 GIST882 cells were transfected with 2 μg of plasmid DNA containing the insert (shPTPN18) or without the insert (shControl) as a negative control, using the Amaxa nucleofection system (Lonza AG) 48 h before imatinib treatment. Transfection efficiency was determined by measuring the cellular *PTPN18* levels using RT–qPCR and western blotting analyses (Supplementary Figure S1B).

Imatinib cytotoxicity assay. The effect of *miR-125a-5p* over-expression or inhibition on imatinib response was evaluated using the WST-1 colorimetric assay (Roche Applied Science, Mannheim, Germany), as previously described (Caramuta *et al*, 2010). After 48 h of transfection, 30 000 cells per well were seeded in 96-well plates and were left either untreated (as mock control) or treated with three different concentrations of imatinib (0.05 μM, 0.1 μM and 0.5 μM for GIST882; 1 μM, 5 μM and 10 μM for GIST48). All experiments were conducted in five wells for each condition and replicated at least three times in independent experiments. The percentage of surviving cells was calculated by comparing the absorbance values of the samples after background subtraction and normalised to the respective cells without imatinib treatment. Imatinib mesylate was kindly provided by Novartis Pharma (Basel, Switzerland).

Target prediction for *miR-125a-5p*. The potential mRNA targets of *miR-125a-5p* were predicted by miRecords (http://mirecords.biolead.org/). The targets that are predicted by at least six programs were chosen for subsequent analyses. Functional annotation and gene ontology analyses were performed by GeneCodis3 (http://genecodis.cnb.csic.es/), using a combination of GO biological process and molecular function parameters.

Western blotting analysis. Cells and frozen tissues were homogenised in NP-40 Lysis buffer (Life Technologies) with addition of protease inhibitor (Complete protease inhibitor cocktail; Roche) and 1 mM of phenylmethanesulfonyl fluoride (Sigma-Aldrich, St Louis, MO, USA). Quantification was done using Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Lysates (60 μg for cells and 100 μg for tissues) were separated by Novex 4%–12% NuPAGE Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes (Life Technologies). After blocking with 5% non-fat milk diluted in TBS, membranes were incubated with anti-PTPN18 antibody (No. 8311, Cell Signaling Technology, Danvers, MA, USA) at 1 : 1000 dilution, followed by anti-rabbit IgG-HRP (1 : 3000; No. 170-6515; Bio-Rad Laboratories, Hercules, CA, USA), or with anti-StARD13 (1 : 200; sc-67843; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by anti-goat IgG-HRP (1 : 5000; 31402; Thermo Scientific). Detection was carried out with Novex ECL HRP chemiluminescent substrate reagent (No. WP20005; Life Technologies). Precision Plus Protein All Blue Standards (No. 161-0373; Bio-Rad Laboratories) was used to determine the relative molecular weights. Protein levels were quantified on immunoblots using the Image Gauge v.4.0 software (FujiFilm Science Lab, Tokyo, Japan). Incubation of the membranes with a GAPDH antibody (sc-47724; Santa Cruz Biotechnology) at 1 : 5000 was performed for normalisation purposes.

Generation of imatinib-resistant clones (GIST882R). GIST882 cells were exposed to 1 μM imatinib for 7 days, leading to the cell death of 60–70% of cells. The remaining cells were harvested and continuously grown in growth media containing 1 μM imatinib. Proteins and RNAs were extracted after 1 month of growth with 1 μM imatinib.

Statistical analysis. All statistical tests were performed in Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) or MS Office Excel 2007 (Albuquerque, NM, USA), unless otherwise stated. Unpaired Student's *t*-test was conducted to compare the miRNA expression levels in different groups, and paired Student's *t*-test was performed to analyse transfection experiments. Association of clinico-pathological features with imatinib response and miRNA expression was evaluated using the Fisher's exact test. The inter-relationship of miRNA expression with survival was studied using the Kaplan–Meier plots, and significant differences between curves were evaluated using the log-rank test. Seventeen cases for which the primary tumour was studied were included in the survival analysis. Recurrences were excluded from the survival analyses as they may carry alterations that are not representative for the primary tumour status and may have different impact on patient survival. Furthermore, two cases with multiple tumours (GIST9 and GIST10) were excluded because of high variation in miRNA expression between individual tumours. Tumours were classified into different groups with high or low expression of each miRNA according to median expression level. All *P*-values obtained in this study were two-tailed, and *P*-values $\leqslant 0.05$ were considered as significant.

RESULTS

Relationship between imatinib response and clinical/genetic features of GISTs. Our sample cohort consisted of 30 GIST samples from 24 patients who received neoadjuvant imatinib treatment (Table 1). Fourteen tumours were imatinib resistant, whereas 16 tumours were imatinib sensitive. GIST9 and GIST10 had four tumours each: three of the tumours in each patient showed progressive growth (imatinib resistant) and one tumour in each patient (GIST9-3 and GIST10-3) partially responded to the treatment (imatinib sensitive). Of the 24 patients, 14 had developed tumour metastasis and the remaining patients had no recurrence or metastasis during the follow-up. Thirteen of the tumours harboured a single non-synonymous mutation in *KIT* and 11 tumours had double *KIT* mutations. The GIST15 tumour harbouring a silent mutation in *KIT* (P585P) was considered as wild type, three tumours had a single mutation in *PDGFRA*, whereas two were wild type for both *KIT* and *PDGFRA*. We evaluated the relationship between imatinib response and clinical/genetic characteristics, including age, gender, metastasis and *KIT* mutational status, in our cohort. As shown in Supplementary Table S3, imatinib resistance was significantly correlated with metastasis ($P = 0.007$; Fisher's exact test) but not with double *KIT* mutations, age or gender.

Distinct miRNA expression patterns in relation to imatinib response and metastasis. We characterised miRNA expression patterns of 17 tumours (10 imatinib sensitive and 7 imatinib resistant) using a microarray-based platform. After normalisation and filtering, 155 miRNAs were included for unsupervised clustering analysis, which is based on similar miRNA expression patterns without prior knowledge of sample identity. The analysis classified the samples into two main subgroups (Figure 1A and Supplementary Figure S2). All imatinib-resistant tumours, except 1 (GIST2), were grouped in cluster 1; while 7 out of the 10 imatinib-sensitive GISTs were found in cluster 2.

Eleven of the 17 GISTs were from patients with metastasis detected either at diagnosis or during follow-up. The other six cases did not have any metastasis during follow-up of at least 25 months (range 25–98); five of which were still alive at the end of follow-up and one died of unrelated cause. The unsupervised clustering analysis grouped 9 out of the 11 metastatic GISTs in cluster 1, and all 6 non-metastatic GISTs in cluster 2 (Figure 1 and Supplementary Figure S2). The three imatinib-sensitive samples (i.e., GIST11, GIST12 and GIST20) that clustered together with

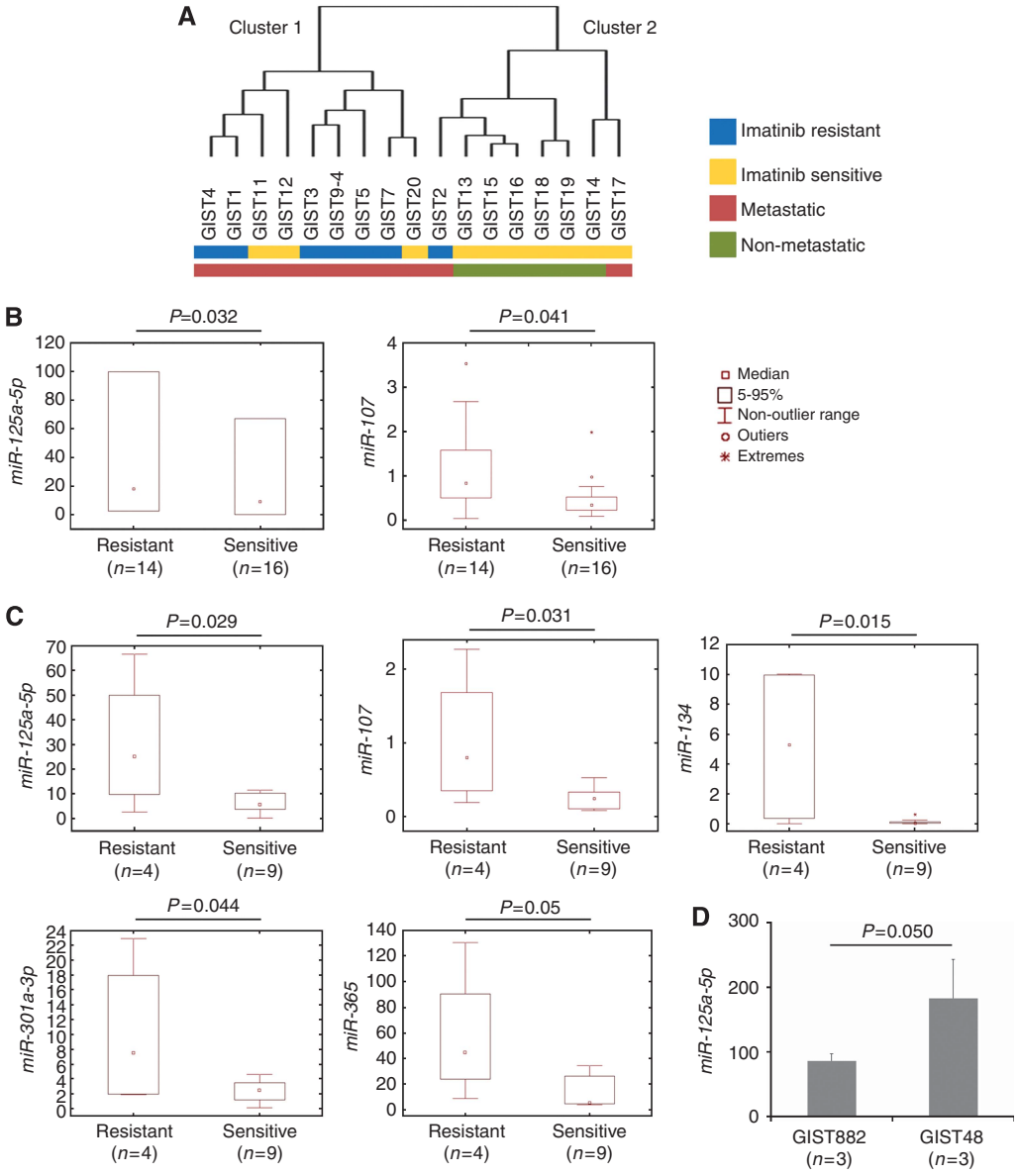

Figure 1. miRNA expression levels in GIST samples. (**A**) Dendrogram based on unsupervised clustering of miRNA expression profiles by microarray in 17 GISTs. (**B**) Box plots illustrate the relative expression levels of *miR-125a-5p* and *miR-107* in 14 imatinib-resistant and 16 imatinib-sensitive GIST specimens regardless of *KIT*/*PDGFRA* mutational status by RT–qPCR after normalisation to *RNU6B*. (**C**) Box plots show the relative expression levels of *miR-125a-5p*, *miR-107*, *miR-134*, *miR-301a-3p* and *miR-365* in four imatinib-resistant and nine imatinib-sensitive tumours among GISTs carrying a single *KIT* mutation. (**D**) Relative *miR-125a-5p* levels in GIST882 (imatinib-sensitive) and GIST48 (imatinib-resistant) cell lines. Differences between groups were calculated using the unpaired Student's *t*-test, and $P \leqslant 0.05$ were considered significant. Error bars refer to s.d. between replicates.

imatinib-resistant tumours had metastasis at diagnosis. Notably, GIST20 showed two distinct areas with tumour necrosis or viable tumour cells in pathological evaluation, indicating that some imatinib-resistant tumour cells were present in this tumour. The survival analysis did not show any significant differences between the two clusters (data not shown).

miRNAs associated with imatinib resistance. Using SAM analysis on the microarray data, we found 27 overexpressed and 17 underexpressed miRNAs with a false discovery rate (FDR) $<15\%$ in the imatinib-resistant GISTs compared with imatinib-sensitive GISTs (Supplementary Table S4).

To verify the array results and to evaluate the significance of the findings, we performed RT–qPCR analyses on 10 selected miRNAs from among the differentially expressed miRNAs in a larger cohort of 30 GIST samples. The selected miRNAs consisted of five overexpressed (*miR-125a-5p*, *miR-301a-3p*, *miR-30c*, *miR-365* and *miR-107*) and five underexpressed miRNAs (*miR-150-3p*, *miR-134*, *miR-1915*, *miR-638* and *miR-1207-5p*) in imatinib-resistant GISTs compared with imatinib-sensitive GISTs. These miRNAs were selected among those with the highest score in the SAM analysis or because of their involvement in drug resistance. We found that the expression of *miR-125a-5p* and *miR-107* was significantly higher (fold changes $= 2.84$ and 2.39, respectively) in the imatinib-resistant tumours ($P = 0.032$ and $P = 0.041$, respectively; *t*-test) (Figure 1B). However, in the two patients (GIST9 and GIST10) with multiple tumours harbouring double mutations in *KIT*, the expression levels of these two miRNAs were not obviously different between the resistant and sensitive tumour pairs (Supplementary Figure S3). The remaining eight miRNAs were not significantly different between the two groups (data not shown).

Given that GISTs harbouring double *KIT* mutations or wild-type *KIT* and *PDGFRA* are known mechanisms of imatinib resistance, we performed an independent evaluation of the samples harbouring only a single *KIT* mutation (all in exon 11). We showed that expression levels of *miR-125a-5p* (4.7-folds; $P=0.029$; *t*-test), *miR-107* (4.1-folds; $P=0.031$), *miR-134* (38.1-folds; $P=0.015$), *miR-301a-3p* (4.4-folds; $P=0.044$) and *miR-365* (4.2-folds; $P=0.05$) were significantly higher in the imatinib-resistant tumours among the GISTs with a single *KIT* mutation (Figure 1C).

We also measured the expression of miRNAs in GIST cell lines. *miR-125a-5p* expression level was higher in the imatinib-resistant cell line (GIST48) and lower in the imatinib-sensitive cell line (GIST882) ($P=0.05$; *t*-test), which corresponded well with its higher level in the imatinib-resistant GISTs. However, the expression pattern of other miRNAs in the GIST cell lines did not corroborate with the expression pattern observed in the clinical samples (Supplementary Figure S4). Given that *miR-125* family members are shown to confer drug resistance (Zhou *et al*, 2010) and regulate cell death pathways in cancer cells (Kim *et al*, 2012), we further investigated the functional role of *miR-125a-5p* in GIST cells.

Effects of *miR-125a-5p* expression on imatinib response in GIST cell lines. Based on our results in clinical samples, we hypothesised that *miR-125a-5p* could act as a modulator of imatinib

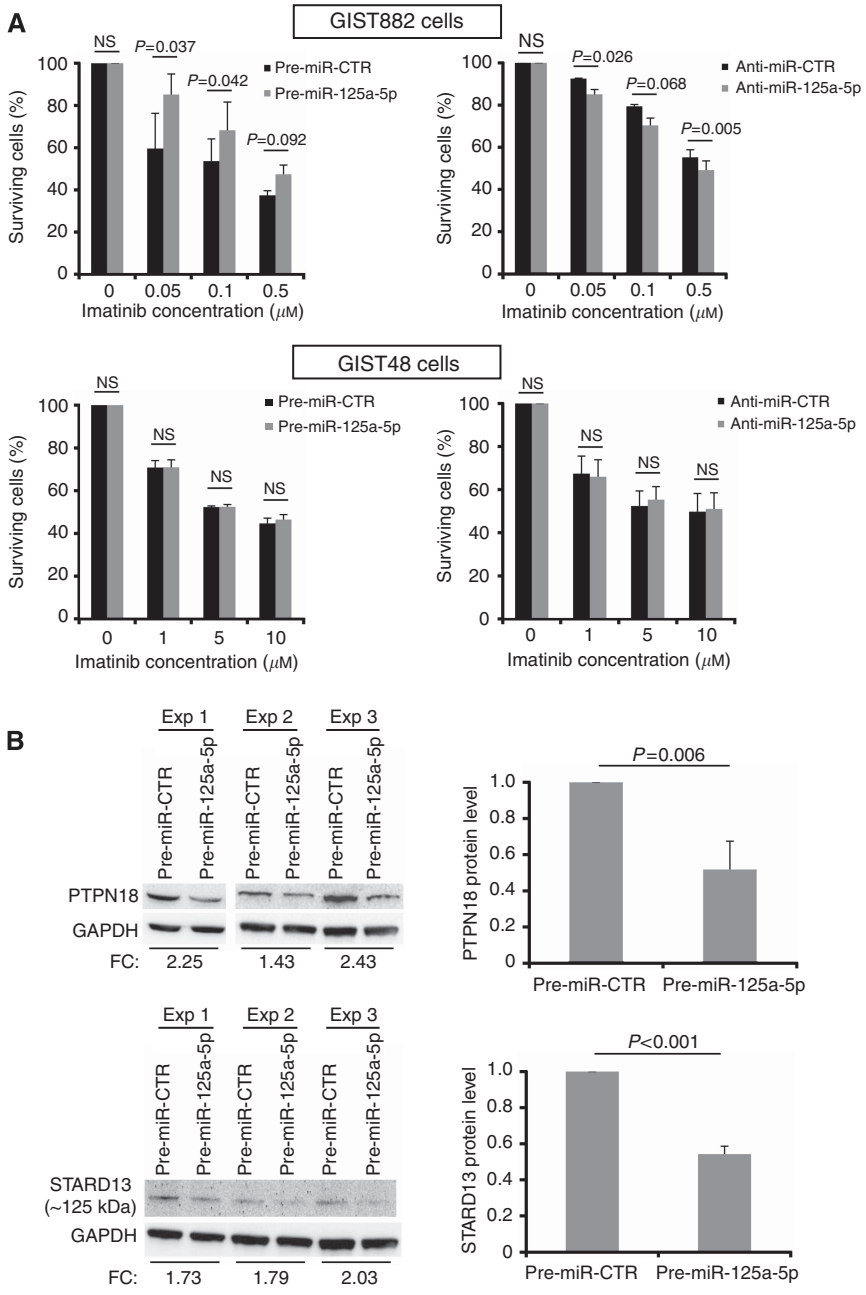

Figure 2. Effects of *miR-125a-5p* modulation on imatinib response and candidate targets in GIST cells. (**A**) Proportion of surviving cells in cultures transfected with pre-miR-125a-5p, anti-miR-125a-5p or their respective negative controls after 24 h of treatment with different concentrations of imatinib, as evaluated by WST-1 assay. (**B**) Western blotting analysis of *miR-125a-5p* candidate targets in GIST882 cells treated with pre-miR-125a-5p or pre-miR-CTR. Western blots show the protein expression levels of PTPN18 and STARD13 in three independent transfection experiments. Bar graphs showing the fold change of protein levels relative to pre-miR-CTR-treated cells after normalisation to GAPDH. Data represent the mean of three independent experiments, and the error bars refer to s.d. from the mean. Statistical differences between the two groups were calculated using the paired Student's *t*-test, and $P\leqslant 0.05$ was considered significant. Abbreviations: FC = fold change; NS = not significant.

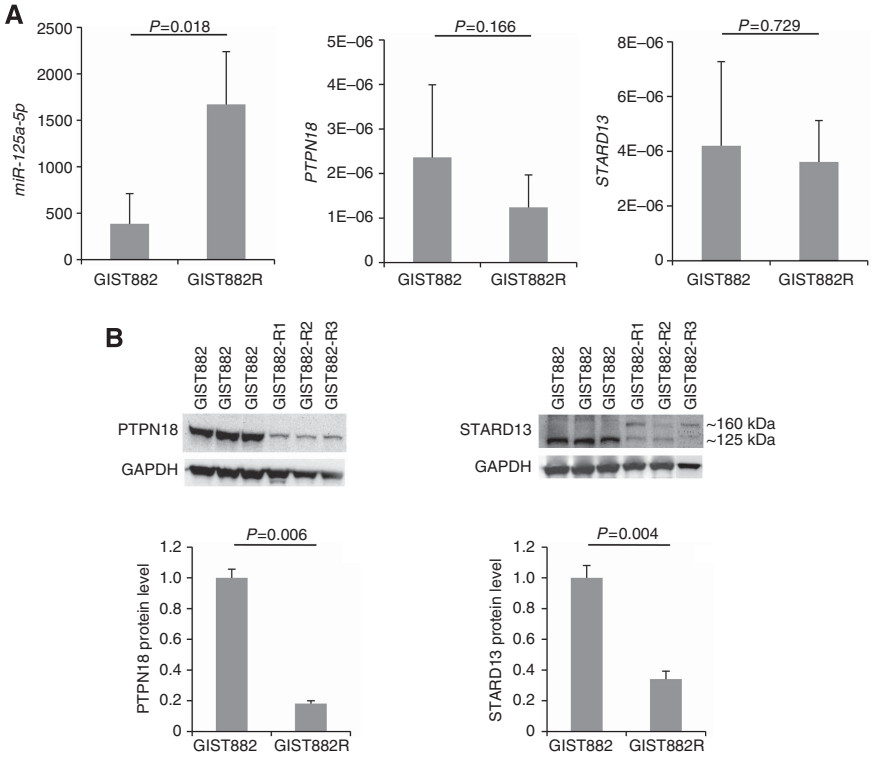

Figure 3. Quantification of *miR-125a-5p*, PTPN18 and STARD13 expression levels in GIST882 parental cells and its imatinib-resistant subclone (GIST882R). (**A**) Quantification of *miR-125a-5p*, *PTPN18* and *STARD13* expression levels by RT–qPCR after normalisation to *RNU6B* or *18S* rRNA. (**B**) Quantification of PTPN18 and STARD13 protein expression levels by western blotting analysis. GAPDH was used as a normalisation control. Data indicate mean values of three independent replicates, and error bars refer to s.d. between replicates. Statistical differences between the two groups were calculated using the paired Student's *t*-test, and $P \leqslant 0.05$ was considered significant.

response among GISTs with a single *KIT* mutations. We next evaluated the functional consequences of *miR-125a-5p* expression on imatinib response in GIST cell lines with a single (GIST882) and double (GIST48) *KIT* mutations. As shown in Figure 2A, overexpression of *miR-125a-5p* in GIST882 cells resulted in a significantly higher (up to 26%) cell viability compared with the cells treated with pre-miR-negative control after 24 h of imatinib treatment, while its suppression caused a significantly lower (up to 9%) cell viability compared with the cells treated with anti-miR-negative control. However, overexpression or suppression of *miR-125a-5p* expression in GIST48 cells did not result in any significant change in cell viability compared with the negative control (Figure 2A). Our results may indicate that *miR-125a-5p* can modulate imatinib response of GIST cells harbouring a single *KIT* mutation but not in GIST cells harbouring *KIT* double mutations. To further support the specificity of *miR-125a-5p* effect on imatinib response, we also evaluated the effects of two additional miRNAs (*miR-944* and *miR-211*), which were not differentially expressed between imatinib-sensitive and -resistant GISTs, as negative controls. Modulation of *miR-944* and *miR-211* did not result in any significant change in imatinib response of GIST882 cells (Supplementary Figure S5).

Analysis and evaluation of *miR-125a-5p* targets in GIST. To better understand the possible role of *miR-125a-5p* on imatinib response, we applied a computational tool (miRecords) to identify the candidate targets of *miR-125a-5p*. The analysis revealed 41 common targets predicted by at least six different target prediction programs (Supplementary Table S5). The predicted targets were then subjected to functional annotation using GeneCodis3. We found several functional groups that were significantly enriched ($P<0.05$), including anti-apoptosis, cell cycle, cell differentiation, transcription, development, protein tyrosine kinase activity, protein phosphorylation and signal transduction (Supplementary Table S6). Among the predicted targets, we selected PTPN18 and STARD13 for further validations because of their involvement in KIT or tyrosine kinase signalling and their regulations by *miR-125* family members (Guo *et al*, 2012; Tang *et al*, 2012). We evaluated the effect of *miR-125a-5p* overexpression on PTPN18 and STARD13 protein levels using western blotting analysis. As shown in Figure 2B, we observed that both PTPN18 ($P=0.006$; *t*-test) and STARD13 ($P<0.001$; *t*-test) protein levels were decreased upon overexpression of *miR-125a-5p* in GIST882 cells, suggesting that they are potential targets of *miR-125a-5p* in GIST cells.

To strengthen the functional evidence of *miR-125a-5p* in imatinib resistance, we generated imatinib-resistant subclones of GIST882 cells and compared the expression level of *miR-125a-5p* and its targets in imatinib-resistant GIST882R subclone with its imatinib-sensitive parental cell line. We observed an increase of *miR-125a-5p* expression in the imatinib-resistant GIST882R cells ($P=0.018$; *t*-test). *PTPN18* and *STARD13* expression levels were not significantly different at mRNA level (Figure 3A), but the protein expression levels of *PTPN18* ($P=0.006$; *t*-test) and *STARD13* ($P=0.004$; *t*-test) were significantly lower in the GIST882R cells compared with its parental cell line (Figure 3B).

We also measured the expression levels of these candidate targets in 27 GIST samples using western blotting analysis. As shown in Figure 4, we observed that PTPN18 protein levels were significantly lower in imatinib-resistant as compared with imatinib-sensitive samples ($P=0.003$; *t*-test) and inversely correlated with *miR-125a-5p* levels ($r=-0.40$; $P=0.036$). STARD13 was not significantly different between imatinib-resistant and -sensitive tumours ($P=0.18$; *t*-test), and no correlation was found with *miR-125a-5p* expression ($r=-0.11$; $P=0.55$).

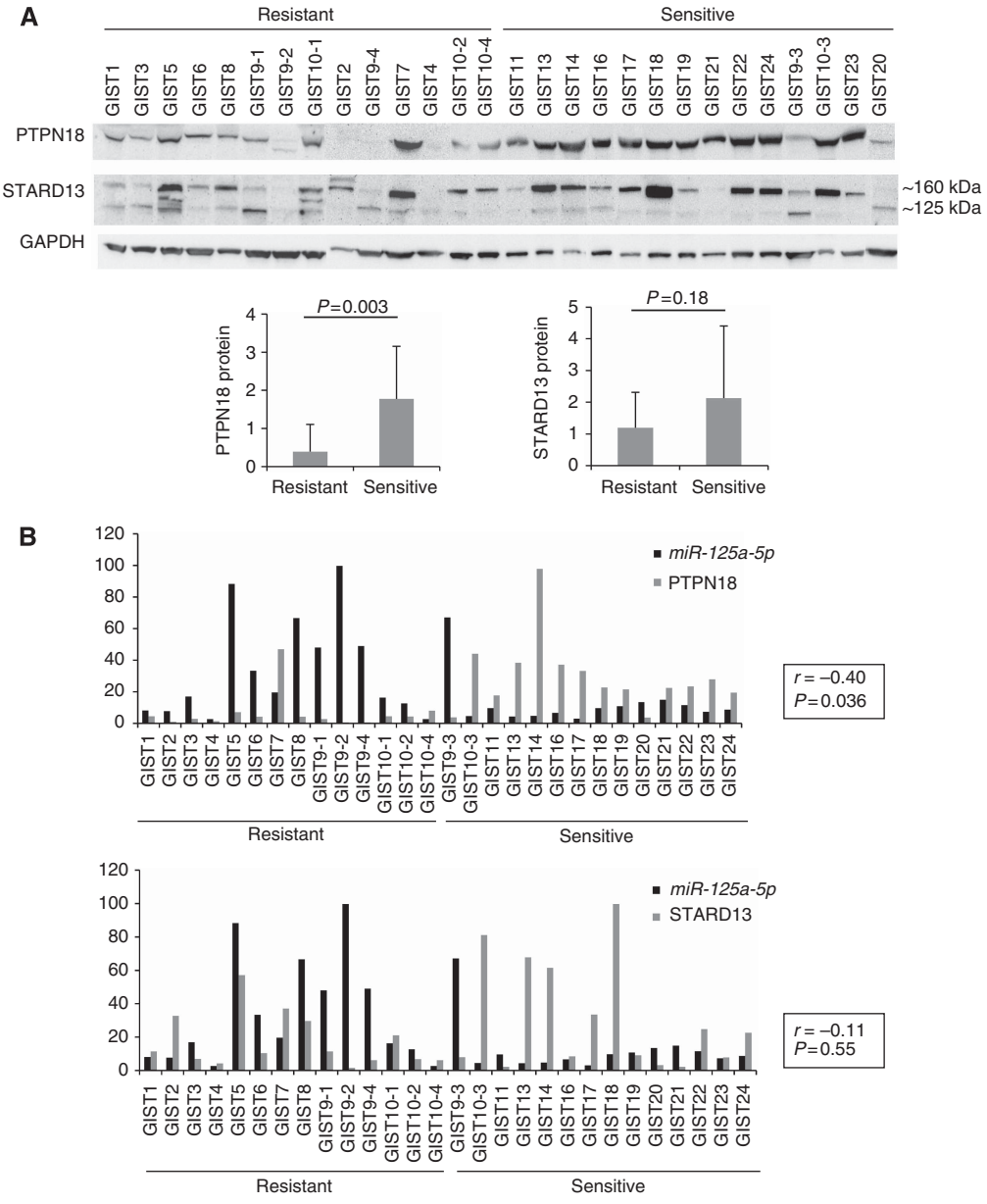

Figure 4. Quantification of PTPN18 and STARD13 protein expression levels in GIST clinical samples by western blotting analysis. (**A**) Western blotting images for PTPN18 and STARD13 (160 and 125 kDa) in clinical samples. Bar graphs showing the protein levels after normalisation to GAPDH. (**B**) Correlation of protein expression levels and *miR-125a-5p* expression levels in imatinib-resistant and -sensitive GISTs. r = correlation coefficient. Statistical differences between the two groups were calculated using the paired Student's *t*-test, and the expression correlation was evaluated by Pearson's correlation analysis. $P \leqslant 0.05$ were considered significant.

To further evaluate the direct functional role of PTPN18 on imatinib response, we determined the effect of PTPN18 suppression on imatinib response in GIST882 cells. As shown in Figure 5, suppression of *PTPN18* in GIST882 cells resulted in a significantly higher cell viability upon imatinib treatment, as compared with the cells treated with control plasmid.

miRNA expression levels are associated with metastasis, *KIT* mutational status and survival. We performed SAM analysis on the microarray data set to identify the most significant miRNAs that could distinguish metastatic tumours from non-metastatic tumours. We found 19 overexpressed and 25 underexpressed miRNAs with a FDR < 15% in the metastatic GISTs compared with the non-metastatic GISTs (Supplementary Table S7). Notably, 8 of the 10 selected miRNAs (*miR-150-3p*, *miR-301a-3p*, *miR-30c*, *miR-107*, *miR-134*, *miR-1207-5p*, *miR-638* and *miR-1915*) chosen for RT–qPCR validation from the SAM analysis of imatinib-resistance *vs* imatinib-sensitive GISTs were also present in the SAM list generated for metastatic *vs* non-metastatic GISTs (Supplementary Tables S4 and S7). In concordance with the microarray results, the expression levels of *miR-150-3p* and *miR-301a-3p* ($P = 0.009$ and $P = 0.041$, respectively; *t*-test) were significantly different between the metastatic and non-metastatic GISTs by RT–qPCR (Figure 6A). We also evaluated whether the miRNA expression levels correlated with metastatic potential. We found significant correlations between the metastatic and non-metastatic tumours with the expression level of *miR-301a-3p*, *miR-150-3p*, *miR-1915* and *miR-1207-5p* (Table 2).

To determine whether *KIT* double mutated tumours differ from *KIT* single mutated tumours based on their miRNA expression levels and to identify the most significant deregulated miRNAs between these two groups, we performed SAM analysis on the

samples harbouring only *KIT* mutations (four double *KIT*-mutated *vs* nine single *KIT*-mutated tumours) in the microarray data set. We found 54 overexpressed and 24 underexpressed miRNAs with a FDR < 15% in the *KIT* double mutated GISTs compared with the single mutated GISTs (Supplementary Table S8). Eight of the differentially expressed miRNAs were also evaluated by RT–qPCR. The analysis revealed that tumours harbouring double *KIT* mutations had significantly lower expression levels of *miR-150-3p* ($P = 0.025$; *t*-test) compared with the tumours carrying a single *KIT* mutation (Figure 6B). Using the Fisher's exact test, we found that the expression levels of *miR-301a-3p* ($P = 0.012$) and *miR-1915* ($P = 0.012$) were significantly correlated with *KIT* mutation status (Table 2).

We also evaluated the association of miRNA expression (based on the RT–qPCR data) with disease-free and overall survival using the Kaplan–Meier survival plots and log-rank analyses. We found low expression of *miR-1915* is correlated with shorter disease-free ($P = 0.003$; log-rank test) and overall ($P = 0.001$; log-rank test) survival of GIST patients (Figure 6C).

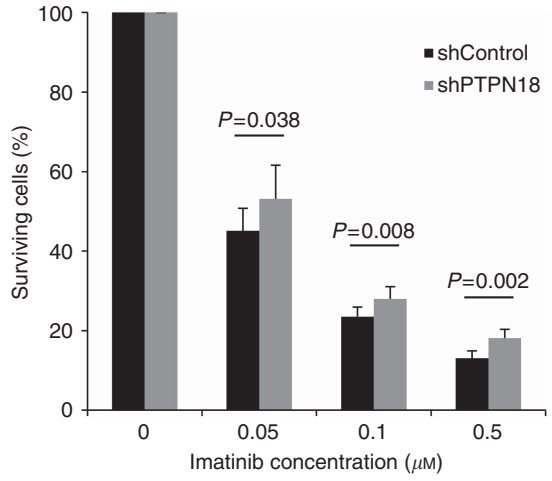

Figure 5. Effect of *PTPN18* silencing on imatinib response in GIST882 cells. Proportion of surviving cells in cultures transfected with short hairpin RNAs against PTPN18 (shPTPN18) or shRNA control plasmid (shControl) after 72 h of treatment with different concentrations of imatinib, as evaluated by WST-1 assay. Statistical differences between the two groups were calculated using the paired Student's *t*-test, and $P \leqslant 0.05$ was considered significant.

DISCUSSION

In this study, we evaluated the clinical impact of miRNA deregulation in imatinib-treated GISTs. We found specific miRNAs associated with imatinib resistance, metastatic disease, *KIT* mutational status and survival in GIST patients.

***miR-125a-5p* modulates imatinib response in GISTs.** We identified a subset of miRNAs significantly associated with imatinib resistance in GIST. Among them, we validated *miR-125a-5p* in a larger cohort of samples using RT–qPCR and functionally characterised its role in GIST cell lines. We show that *miR-125a-5p* expression modulates imatinib responsiveness in the single *KIT*-mutated GIST882 cells but not in the double *KIT*-mutated GIST48 cell line. Given that complete genetic changes are not available for both cell lines, it is possible that other genetic factor(s) (besides *KIT* mutation) may contribute to the different imatinib response observed in the two cell lines. Furthermore, *miR-125a-5p* expression was not different between the sensitive and

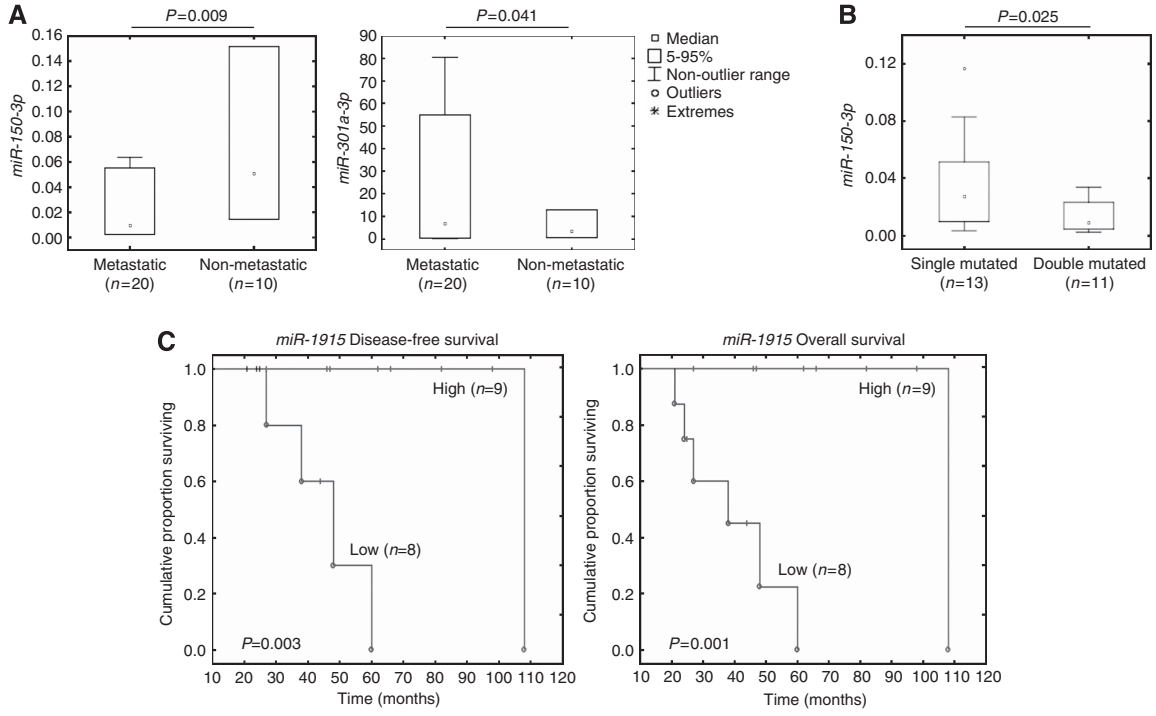

Figure 6. RT–qPCR analysis of miRNA expression levels in relation to metastasis, *KIT* mutational status and survival in GISTs. Box plots show relative expression levels of (**A**) *miR-150-3p* and *miR-301a-3p* in 20 cases of metastatic and 10 non-metastatic GISTs, and (**B**) *miR-150-3p* in 13 single and 11 double *KIT*-mutated tumours. Comparisons between groups were calculated using the unpaired Student's *t*-test, and $P \leqslant 0.05$ were considered significant. (**C**) Kaplan–Meier curves show significant association of *miR-1915* expression with disease-free survival and overall survival in GIST patients. Differences in survival were calculated using the log-rank test. High and low expression levels refer to above and below the median level of all tumours.

Table 2. Associations of microRNA expression levels with metastasis or *KIT* mutational status

	Metastasis			*KIT* mutation		
	Metastatic	Non-metastatic		Single	Double	
Parameter	($n=20$)	($n=10$)	*P*-value[a]	($n=13$)	($n=11$)	*P*-value[a]
miR-301a						
High	13	2	**0.050**	3	9	**0.012**
Low	7	8		10	2	
miR-30c						
High	10	5	1.000	5	7	0.414
Low	10	5		8	4	
miR-134						
High	12	3	0.245	6	6	1.000
Low	8	7		7	5	
miR-1915						
High	6	9	**0.005**	10	2	**0.012**
Low	14	1		3	9	
miR-125a-5p						
High	11	4	0.700	4	8	0.099
Low	9	6		9	3	
miR-365						
High	10	5	1.000	6	5	1.000
Low	10	5		6	6	
miR-107						
High	11	4	0.700	5	7	0.414
Low	9	6		8	4	
miR-150-3p						
High	6	9	**0.005**	8	3	0.123
Low	14	1		5	8	
miR-638[b]						
High	6	6	0.680	7	3	0.387
Low	8	4		5	6	
miR-1207-5p[b]						
High	4	8	**0.036**	8	2	0.080
Low	10	2		4	7	

Note: High and low refer to above and below the median expression level of all tumours.
[a]*P*-values were determined by the two-tailed Fisher's exact test. $P \leqslant 0.05$ are indicated in bold.
[b]Only 24 cases were analysed by RT–qPCR.

resistant tumours of the two patients with multiple tumours harbouring secondary *KIT* mutation. Together, our data suggest the role of miRNA(s) as an alternative resistance mechanism to second site *KIT* mutations in GIST.

Aberrant expression of *miR-125a-5p* has been reported in several cancer types (Guo *et al*, 2009; Wang *et al*, 2009). Low *miR-125a-5p* expression is associated with more aggressive disease and poorer survival in gastric cancer (Nishida *et al*, 2011). Decreased expression of *miR125a-5p* is also associated with docetaxel resistance in human breast cancer cell lines (Kastl *et al*, 2012). Our findings support the notion that *miR-125a-5p* may have tumour-promoting or -suppressing functions depending on the cellular contexts. Several studies have reported diverse functions of *miR-125* family members in different cell types as reviewed by Sun *et al* (2013).

In addition to *miR-125a-5p*, other *miR-125* family members were shown to induce drug resistance (Zhou *et al*, 2010) and regulate cell death pathways, such as NF-κB (Kim *et al*, 2012) and p53 (Leotta *et al*, 2014). Functionally, *miR-125a-5p* has a role in cell proliferation, migration, invasion and apoptosis in different cancer cell types (Wang *et al*, 2009; Nishida *et al*, 2011). Using a computational approach, we found that the predicted targets of *miR-125a-5p* are functionally associated with several biological processes, such as anti-apoptosis, cell cycle, cell differentiation, signal transduction and protein phosphorylation, which have been implicated in drug resistance of different cancer types. In line with the functional annotation of the *miR-125a-5p* predicted targets, deregulation of genes involved in cell cycle control, apoptosis and muscle differentiation has been associated with imatinib response in GIST patients (Agaram *et al*, 2007; Romeo *et al*, 2009).

Among the predicted targets, we chose PTPN18 and STARD13 as candidate targets of *miR-125a-5p*. We show that both candidates are regulated by *miR-125a-5p* in GIST cells, and we further validated the association of PTPN18 expression with *miR-125a-5p* expression and imatinib response in GIST clinical samples and cell lines. *PTPN18* is an interesting candidate for several reasons. First, it is involved in the negative feedback mechanisms controlling the Bcr-Abl fusion tyrosine kinase-signalling network by inhibiting the phosphorylation of Src family kinase (Rubbi *et al*, 2011). Second, PTPN18 can induce actin cytoskeleton reorganisation (Shiota *et al*, 2003), suggesting a plausible explanation of the morphological changes observed in the imatinib-resistant GIST cells (Mahadevan *et al*, 2007). Third, altered phosphorylation of tyrosine kinases is an alternative mechanism of imatinib resistance in GIST (Takahashi *et al*, 2013), suggesting the possible involvements of protein phosphatases in imatinib resistance of GIST. Here, we propose the role of PTPN18 in imatinib response through *miR-125a-5p* regulation, and this regulation may involve in imatinib resistance as an alternative mechanism to *KIT* second site mutations in GIST.

Metastasis-associated miRNAs in GIST. We show associations of two miRNAs (*miR-301a-3p* and *miR-150-3p*) with metastasis. In concordance with our results in GIST, *miR-301a-3p* overexpression was found in metastatic breast cancer (Shi *et al*, 2011). Functionally, *miR-301a-3p* has been demonstrated to promote cell growth, migration and invasion in breast cancer cells (Shi *et al*, 2011) and hepatocellular carcinoma cells (Zhou *et al*, 2012). These findings support the role of *miR-301a-3p* as an oncogene in progression and development of metastasis in various cancer types. On the other hand, very little is known about *miR-150-3p*. Interestingly, its upregulation was shown in colorectal cancers with liver metastasis (Lin *et al*, 2011).

GISTs with a single or double *KIT* mutations. Despite numerous studies revealed that double mutations confer acquired imatinib resistance in GISTs, we (in this study) and others (Antonescu *et al*, 2005; Wardelmann *et al*, 2006) found a proportion (40–53%) of imatinib-resistant GISTs harbouring a single *KIT* mutation, suggesting the involvement of alternative resistance mechanisms. Furthermore, we showed that several miRNAs were significantly deregulated between the GISTs with a single mutation and double mutations. Those differentially expressed miRNAs may be involved in partly distinct pathways in GISTs with single and double *KIT* mutations. Although Allander *et al* (2001) reported homogeneous gene expression profiles in GISTs with different *KIT* mutations, distinct clusters were also observed among the GISTs with *KIT* mutations (Allander *et al*, 2001; Yamaguchi *et al*, 2008), suggesting subgroups of GIST patients harbouring *KIT* mutations. Because of the lack of information on secondary mutations in those studies, we could not determine whether the different clusters observed in the gene expression profiles reflect the single and double mutations in *KIT*. In line with the *KIT* mutations in GIST, acute myeloid leukemias with single or double *CEBPA* mutations have distinct gene expression profiles and different clinical outcomes (Wouters *et al*, 2009). Further investigations are warranted to determine the biological and clinical differences between GISTs with single and double *KIT* mutations.

Prognostic miRNAs in GIST patients. We identified *miR-1915* as a candidate prognostic marker for survival in GIST under imatinib therapy. Interestingly, *miR-1915* expression is also significantly correlated with metastasis and double *KIT* mutation (Table 2). Recently, Niinuma *et al* (2012) reported that *miR-196a* overexpression was significantly associated with high-risk grade, metastasis and poor survival in GIST, while Choi *et al* (2010) revealed a different subset of miRNAs associated with tumour risk. The discrepancy in the findings is likely due to sample selection. There is no information given whether the studied cohorts in both studies were treated with imatinib on a neoadjuvant basis.

In summary, we report the identification of miRNAs associated with imatinib resistance, metastasis, *KIT* mutational status and survival in GIST. Our findings suggest that differential expression of these miRNAs may contribute to the prognosis of GIST patients treated with imatinib preoperatively. Importantly, we provide functional evidence that *miR-125a-5p* can modulate imatinib response in single *KIT*-mutated GIST cells through PTPN18 regulation, suggesting the prognostic impact of *miR-125a-5p* and PTPN18 for GIST patients under neoadjuvant imatinib therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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