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MiR-17-92 and miR-221/222 cluster members target KIT and ETV1 in human gastrointestinal stromal tumours

C M M Gits[1], P F van Kuijk[1], M B E Jonkers[1], A W M Boersma[1], W F van IJcken[2], A Wozniak[3], R Sciot[4], P Rutkowski[5], P Schöffski[3], T Taguchi[6], R H J Mathijssen[1], J Verweij[1], S Sleijfer[1], M Debiec-Rychter[7] and E A C Wiemer*[,1]

[1]*Department of Medical Oncology, Erasmus University Medical Center – Erasmus MC Cancer Institute, Dr Molewaterplein 50, 3015 GE, Rotterdam, The Netherlands;* [2]*Center for Biomics, Erasmus University Medical Center, Dr Molewaterplein 50, 3015 GE, Rotterdam, The Netherlands;* [3]*Laboratory of Experimental Oncology, Departments of Oncology and General Medical Oncology, KU Leuven and University Hospitals Leuven, Herestraat 49, B – 3000, Leuven, Belgium;* [4]*Department of Pathology, KU Leuven and University Hospitals Leuven, Minderbroedersstraat 12, B – 3000 Leuven, Belgium;* [5]*Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, 5 Roentgena Street, 02-781 Warsaw, Poland;* [6]*Division of Human Health and Medical Science, Graduate School of Kuroshio Science, Kochi University, Nankoku, Kochi 783-8505, Japan and* [7]*Department of Human Genetics, KU Leuven and University Hospitals Leuven, Herestraat 49, B - 3000 Leuven, Belgium*

Background: Gastrointestinal stromal tumours (GIST) are characterised by high expression of KIT and ETV1, which cooperate in GIST oncogenesis. Our aim was to identify microRNAs that are deregulated in GIST, have a role in GIST pathogenesis, and could potentially be used as therapeutic tool.

Methods: Differentially expressed microRNAs between primary GIST ($n=50$) and gastrointestinal leiomyosarcomas (GI-LMS, $n=10$) were determined using microarrays. Selected microRNA mimics were transfected into GIST-882 and GIST-T1 cell lines to study the effects of microRNA overexpression on GIST cells. Luciferase reporter assays were used to establish regulation of target genes by selected microRNAs.

Results: MiR-17-92 and miR-221/222 cluster members were significantly ($P<0.01$) lower expressed in GIST *vs* GI-LMS and normal gastrointestinal control tissues. MiR-17/20a/222 overexpression in GIST cell lines severely inhibited cell proliferation, affected cell cycle progression, induced apoptosis and strongly downregulated protein and – to a lesser extent – mRNA levels of their predicted target genes *KIT* and *ETV1*. Luciferase reporter assays confirmed direct regulation of *KIT* and *ETV1* by miR-222 and miR-17/20a, respectively.

Conclusion: MicroRNAs that may have an essential role in GIST pathogenesis were identified, in particular miR-17/20a/222 that target *KIT* and *ETV1*. Delivering these microRNAs therapeutically could hold great potential for GIST management, especially in imatinib-resistant disease.

Gastrointestinal stromal tumours (GIST) are the most common mesenchymal tumours of the gastrointestinal (GI) tract. GIST originate from the interstitial cells of Cajal (ICC) or from their precursor stem cells (Hirota *et al*, 1998; Kindblom *et al*, 1998; Sircar *et al*, 1999). ICC function as pacemaker cells of the GI tract inducing the peristaltic movements. They show immunophenotypical

*Correspondence: Dr EAC Wiemer; E-mail: e.wiemer@erasmusmc.nl

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and ultrastructural features of both smooth muscle and neuronal differentiation and reside between the circular and longitudinal muscle layers of the GI tract (Tornillo and Terracciano, 2006; Liegl-Atzwanger *et al*, 2010; Corless *et al*, 2011). Therefore, GIST were initially commonly classified as gastrointestinal leiomyosarcomas (GI-LMS).

In contrast to GI-LMS and other soft tissue sarcomas, a common (>95%) feature of GIST is the expression of v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue (KIT), a tyrosine kinase receptor (Hirota *et al*, 1998; Sarlomo-Rikala *et al*, 1998; Corless *et al*, 2011). KIT expression is also a characteristic of ICC and other cell types such as hematopoietic stem cells, melanocytes, mast cells and germ cells. In all these cells, ligand-dependent activation of KIT signalling is important for proliferation, differentiation and survival. In addition to KIT expression, spontaneous gain-of-function mutations in *KIT* (~80%) or platelet-derived growth factor receptor alpha (*PDGFRA*; ~10%) are frequently found in GIST. These mutations, which are mutually exclusive and have similar biological functions, are considered oncogenic drivers of GIST (Hirota *et al*, 1998; Lasota *et al*, 1999; Heinrich *et al*, 2003; Lasota and Miettinen, 2006; Liegl-Atzwanger *et al*, 2010; Corless *et al*, 2011). The mutant receptors display constitutive kinase activity, which is independent of growth factor binding. Thereby downstream signalling pathways such as the RAS-RAF-MAPK , PI3K-AKT pathways and the STAT3 transcription factor are activated, giving rise to increased cell proliferation and survival.

In a small subset of GIST without mutations in *KIT* and *PDGFRA* (wild-type (WT)-GIST), other genes are thought to have a role in the tumorigenesis. Mutated genes found in WT-GIST include *BRAF* (Agaram *et al*, 2008; Agaimy *et al*, 2009; Hostein *et al*, 2010) and succinate dehydrogenase subunits B, C and D (*SDHB*, *SDHC* and *SDHD*) (Pasini *et al*, 2008; Janeway *et al*, 2011).

Recently, another factor was identified to have a role in the tumour biology of GIST. ETV1 cooperates with KIT and is essential both in normal ICC development as well as in GIST oncogenesis (Chi *et al*, 2010). ETV1 is a member of the ETS family of transcription factors, which are frequently deregulated in human malignancies and have a characteristic highly conserved DNA-binding (ETS) domain (Seth and Watson, 2005). Similar to KIT, ETV1 is highly expressed in ICC and GIST, where it is a master regulator of ICC/GIST-specific transcriptional networks. Normal WT-KIT and ETV1 levels will ensure natural ICC development. Mutated KIT prolongs ETV1 protein stability, thereby leading to overactivation of ETV1-mediated transcriptional programmes, which induce hyperplasia and finally GIST formation (Chi *et al*, 2010).

Although many aspects of the tumour biology of GIST have been elucidated, some issues remain. One of these is the underlying mechanism causing overexpression of KIT and ETV1. As KIT (and most likely ETV1) overexpression is rarely caused by gene amplification, it is postulated that other mechanisms such as transcriptional or translational regulation, or processes that act on RNA stability may be involved (Tabone *et al*, 2005). MicroRNAs (miRNAs) may influence KIT and/or ETV1 expression as they are non-protein coding RNAs that bind to (partially) complementary sites in 3′-UTRs of target mRNAs causing translational inhibition and/or mRNA degradation. In recent years, miRNAs have been implicated in a wide range of important cellular processes like proliferation, differentiation, apoptosis and stem cell maintenance. Their expression is cell type specific and often deregulated in disease, including cancer, in which miRNAs can function as oncogene or tumour suppressor (Chen, 2005; Zhang *et al*, 2007; Di Leva and Croce, 2010). miRNA expression patterns of GIST have been associated with genomic changes and biological behaviour (Choi *et al*, 2010; Haller *et al*, 2010). Downregulation of miR-494, as well as the miR-221/222 cluster, has been correlated with KIT protein expression in GIST (Kim *et al*, 2011; Koelz *et al*, 2011).

In this study, we aimed to get more insight into the potential role of miRNAs in GIST. We compared the miRNA expression profiles of GIST and GI-LMS samples to identify miRNAs that could be of biological importance in GIST. We focused on the differentially expressed miR-17-92 and miR-221/222 clusters, which have relatively low expression levels in GIST and potentially target both KIT and ETV1.

MATERIALS AND METHODS

Tumour samples. Frozen specimens of 50 primary GIST and 10 GI-LMS were obtained from the tissue banks of the Department of Pathology of University Hospital of Leuven, Belgium, and the Department of Soft Tissue/Bone Sarcoma and Melanoma, Institute of Oncology, Warsaw, Poland. All patients gave written informed consent, and the approval was obtained from the University Hospital Leuven ethics committee (ML7481) and Institute of Oncology, Warsaw, Poland. Diagnosis of GI-LMS was based on morphological features and immunophenotype (desmin and h-caldesmon immunopositive; S100, CD34, CD117/KIT and DOG1 immunonegative). GIST diagnosis was based on histological features and immunostaining (CD117/KIT and DOG1 immunopositive) and the presence of KIT or PDGFRA mutations. Tumour samples contained >80% tumour cells. In addition, frozen specimens of five normal gastric and five normal small intestinal control tissues were obtained from the Erasmus MC Tissue Bank. The study was approved by the Medical Ethical Review Board of the Erasmus University Medical Center.

RNA isolation. Total RNA was isolated by standard RNAbee (Tel Test Inc., Friendswood, TX, USA) extraction. In short, frozen tissues were homogenised in RNAbee after which chloroform was added. Phase separation by centrifugation was followed by RNA precipitation using isopropanol. The RNA pellet was washed twice in 75% EtOH and dissolved in nuclease-free water. RNA concentration and quality were tested on Nanodrop-1000 (Nanodrop Technologies, Wilmington, DE, USA).

miRNA microarray. One microgram of total RNA was fluorescently labelled with Cy3 using the Kreatech ULS aRNA labelling kit (Kreatech Diagnostics, Amsterdam, The Netherlands) following the standard protocol. Labelled RNA was hybridised in a Tecan HS4800 hybridisation station to in-home spotted array slides (Pothof *et al*, 2009) with locked nucleic acid (LNA)-modified oligonucleotide capture probes (Exiqon, Vedbaek, Denmark) capable of detecting 725 human miRNAs in duplicate. Slides were scanned using a Tecan LS reloaded scanner, and median spot intensity was determined using ImaGene software (BioDiscovery Inc., Hawthorne, CA, USA). After background subtraction, values were quantile normalised by *R* software, bad spots were deleted and duplicate spots were averaged. For each value, the ratio to the geometric mean of the miRNA was log2 transformed. These values were used to determine differentially expressed miRNAs and to perform statistical testing by a two-sample *t*-test in BRB-Array Tools (Microsoft Excel plug-in). Hierarchical clustering analyses were performed in Spotfire software (Spotfire DecisionSite 9.1, Tibco Software, Somerville, MA, USA). Boxplots were created in SPSS software (IBM Corporation, Armonk, NY, USA).

qPCR analysis of miRNAs. RNA (50 ng) was reverse transcribed by using specific miRNA primers from the TaqMan MicroRNA Assays for hsa-miR-17, hsa-miR-20a, hsa-miR-222 and RNU48, and reagents from the TaqMan MicroRNA Reverse Transcription Kit (both Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) according to the manufacturer's protocol. The resulting cDNA was used in a quantitative real-time PCR (qPCR) with the primer/probe mix from the TaqMan MicroRNA Assay

together with the TaqMan Universal PCR Master Mix No AmpErase UNG using the 7500 Fast Real-Time PCR system (all Applied Biosystems) according to the manufacturer's protocol. qPCR data were analysed using SDS software (Applied Biosystems). MiRNA expression was normalised using RNU48 expression and the comparative C_T-method (Schmittgen and Livak, 2008).

qPCR analysis of mRNAs. RNA (1 μg) was reverse transcribed using the TaqMan Reverse Transcription reagents (Applied Biosystems) according to the manufacturer's protocol. The resulting cDNA was used to perform qPCR using the primer/probe mix from the TaqMan Gene Expression Assays of human *KIT* and *ETV1* (with exon-spanning probes) and TaqMan Universal PCR Master Mix using the 7500 Fast Real-Time PCR system (all Applied Biosystems) according to the manufacturer's protocol. Three housekeepers (*GAPDH*, *HPRT* and *HMBS*) were analysed for normalisation purposes using the comparative C_T-method (Schmittgen and Livak, 2008). qPCR data were analysed using SDS software (Applied Biosystems).

Cell culture. The GIST-T1 cell line was provided by Dr T. Taguchi (Kochi University, Japan). This *KIT* exon 11 mutant cell line is characterised by a heterozygous deletion of 57 bases (Taguchi *et al*, 2002). The GIST-882 was kindly provided by Dr J. Fletcher (Dana-Farber Cancer Institute, Boston, MA, USA). This cell line is characterised by a *KIT* exon 13 missense mutation, resulting in a single amino-acid K642E substitution in the proximal part of the cytoplasmic split tyrosine kinase domain (Tuveson *et al*, 2001). Both cell lines were cultured in RPMI 1640 (Invitrogen, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum, at 37 °C, 5% CO_2. The imatinib-resistant GIST-T1-R cell line was provided by Dr S. Bauer (West-German Cancer Center, University Duisburg-Essen, University Hospital Essen, Germany). The GIST-T1-R is derived from GIST-T1 and contains secondary KIT exon 17 mutations causing imatinib resistance. GIST-T1-R is cultured in RPMI/HAM-F12 (Invitrogen), supplemented with 20% fetal bovine serum, in the presence of 1 μM imatinib, at 37 °C, 5% CO_2.

Mimic transfections. MiRIDIAN microRNA mimics (Thermo Scientific, Etten-Leur, The Netherlands) of hsa-miR-17, hsa-miR-20a, and hsa-miR-222 and miRIDIAN microRNA Mimic Negative Control #1 (Thermo Scientific) were transfected in a final concentration of 50 nM using DharmaFECT1 transfection reagent (Thermo Scientific) 24 h after cell seeding. For both cell lines, transfection efficiency was optimised to >95% using fluorescent mimics, and increased expression levels of miR-17, miR-20a and miR-222 in transfected cells were verified by qPCR.

Cell proliferation assay. Cell proliferation was monitored using a sulforhodamine B (SRB) assay at 24 h, 48 h, 72 h and 96 h post transfection (Keepers *et al*, 1991). In short, cells were fixed by 10% TCA in PBS, washed with half warm tap water, stained by 0.4% SRB in 1% acetic acid for 15 min, washed in 1% acetic acid and dried. Colour was released from the cells by 10 mM Tris-base, after which A540 nm was measured on a spectrophotometer. Three independent experiments were performed and representative graphs are shown.

Cell cycle assay. Cells were harvested by trypsinisation 72 h post transfection, fixed in 70% ethanol for 15 min, washed in PBS and resuspended in PBS/RNAse/propidium iodide (PI) (20 μg ml^{-1} RNAse, 50 μg ml^{-1} PI, 1% fetal bovine serum). PI staining was FACS-analysed using a 488 nm laser with emission filters LP655 and BP695/40 (flowcytometer FACS Aria III, BD Biosciences, Breda, The Netherlands) and analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Experiments were performed three times and representative figures are shown.

Apoptosis assay. Cells were collected by trypsinisation 72 h post transfection and stained with FITC-Annexin V and PI by using the FITC-Annexin V Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's protocol. In short, cells were washed in PBS and resuspended in 1 × binding buffer. FITC-Annexin V and PI were added to the solution (~10^5 cells) and incubated on ice for 15 min in the dark. More binding buffer was added before analysis using a 488 nm laser with emission filters LP520 nm and BP530/30 nm (flowcytometer FACS Aria III, BD Biosciences). Three independent experiments were performed and representative figures are shown.

Protein extraction. Cells were collected in MCB lysis buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 10% glycerol, 1% NP-40, 0.5% Na-deoxycholate, 20 mM NaF) supplemented with a cocktail of protease and phosphatase inhibitors. Lysates were thoroughly vortexed and further lysed by two subsequent freeze-thaw cycles using liquid nitrogen. Cell debris was spun down, and protein concentration was determined using a Bradford assay (BioRad, Veenendaal, The Netherlands).

Western blotting. Thirty micrograms of total protein was subjected to SDS–PAGE. Proteins were transferred to a PVDF membrane followed by blocking of the membrane in 5% non-fat dry milk in PBS-Tween (PBS, 0.05% Tween 20) to prevent non-specific antibody binding. Primary and secondary antibody incubations were carried out in the same buffer using the anti-CD117/c-kit (YR145, rabbit monoclonal, 1 : 500, Cell Marque, Rocklin, CA, USA), anti-ER81/ETV1 (ab81086, rabbit polyclonal, 1 : 1000, Abcam, Cambridge, UK) and anti-β-Actin (AC-15, mouse monoclonal, 1 : 5000, Sigma-Aldrich, Zwijndrecht, The Netherlands) as a loading control. As secondary antibody HRP-conjugated goat-anti-mouse (1 : 10000, Santa Cruz Biotechnology, Heidelberg, Germany) or goat-anti-rabbit (1 : 10 000, Jackson Immunoresearch, Suffolk, UK) antibodies were used. Antibody incubations were followed by enhanced chemoluminescence (Supersignal West Pico Chemiluminescent Substrate, Thermo Scientific) and visualised on the film (Amersham Hyperfilm ECL, GE Healthcare, Diegem, Belgium).

Cloning. Fragments of the 3′-UTR of *ETV1* (ETV1short: 708 bp fragment, ETV1long: 3721 bp fragment) and *KIT* (KITshort: 1263 bp fragment, KITlong: 2055 bp fragment) were PCR amplified from human genomic DNA (Promega, Leiden, The Netherlands) introducing a *Xho*I (5′-end) and a *Not*I site (3′-end). The PCR products were cloned in PCR-Blunt (Invitrogen), followed by a *Xho*I and *Not*I restriction and ligation in the psiCHECK-2 vector (Promega) behind a *Renilla* luciferase gene. The psiCHECK-2 vector also contains a firefly luciferase gene, which was used for normalisation. The resulting constructs are psiCHECK2/ETV1-short, psiCHECK2/ETV1long, psiCHECK2/KITshort and psiCHECK2/KITlong. Site-directed mutagenesis (QuikChange II XL, site directed mutagenesis kit, Agilent Technologies, Amsterdam, The Netherlands) was performed to mutate miR-17/20a and miR-222 target sites in the 3′-UTR of KIT and ETV1 (Supplementary Figure 1). Primer sequences used for cloning and mutagenesis are listed in Supplementary Table 1.

Luciferase assay. PsiCHECK-2 constructs were transfected using Fugene HD transfection reagent (Promega) 24 h after cell seeding, according to recommendations by the manufacturer. The next day, cells were transfected with hsa-miR-17, hsa-miR-20a and hsa-miR-222 miRIDIAN mimics, a mixture of these three mimics, and miRIDIAN microRNA Mimic Negative Control #1 (Thermo Scientific) to a final concentration of 50 nM using DharmaFECT1 transfection reagent (Thermo Scientific). After 48 h, protein lysates were prepared in which firefly and *Renilla* luciferase activities were quantified using the Dual Luciferase Reporter Assay System (Promega). *Renilla* luciferase expression was normalised on the

firefly luciferase expression, and relative luciferase signals of duplicates were averaged. Two independent experiments were normalised, after which, average and standard deviations were calculated. Significant differences in luciferase activity were determined using two-sample *t*-tests.

RESULTS

Identification of differentially expressed microRNAs between GIST and GI-LMS. In order to identify miRNAs that are specifically deregulated in GIST, we compared the miRNA expression profile of primary GIST ($n=50$) and GI-LMS ($n=10$). Patients' and tumours' characteristics are listed in Table 1. The GIST group contained both *PDGFRA* ($n=19$) and *KIT* ($n=31$) mutants, obtained from different anatomical sites (gastric and non-gastric), which were clinically overtly malignant or had variable risks of relapse (Table 1) (Miettinen and Lasota, 2006). The miRNA expression was analysed on a microarray platform containing LNA capture probes of which 725 hybridize to annotated human miRNAs. A considerable number of miRNAs were differentially expressed ($n=219$; $P<0.05$) (Supplementary Table 2). The most significant differentially expressed miRNAs between GIST and GI-LMS (two-sample *t*-test, $P<0.005$, corrected for multiple testing $P<0.03$) were used in a supervised hierarchical clustering (Figure 1). This analysis separates the tumours in two main groups with all GI-LMS clustering in one branch, together with 10 GIST. Neither mutation type, tumour location, origin of samples (Warsaw *versus* Leuven), nor relapse risk could explain why these 10 GIST are apparently molecularly distinct and resemble the GI-LMS more closely than the other GIST. It may be that the GIST/GI-LMS clustering has a biological basis implying these tumours share to some extent a common biology.

MiR-17-92 and miR-221/222 cluster members distinguish GIST from GI-LMS. Strikingly, the list of differentially expressed miRNAs between GIST and GI-LMS contained a number of miRNA clusters, for example, members of the miR-17-92 cluster (i.e., miR-17, miR-18a, miR-19a and miR-20a) and the miR-221/222 cluster (all $P<0.01$, corrected for multiple testing $P<0.05$). Of note, miR-494, which was previously found to inversely correlate with KIT expression (Kim *et al*, 2011), was not differentially expressed between our groups. We focused on the miR-17-92 and miR-221/222 clusters as the latter is known for its association with KIT expression in GIST (Koelz *et al*, 2011), whereas the miR-17-92 cluster is notorious for its oncogenic role in many tumour types (He *et al*, 2005; Volinia *et al*, 2006; Guo *et al*, 2009; Schulte *et al*, 2010). The expression levels of these cluster members were significantly lower in GIST compared with GI-LMS (Figure 2A). Moreover, these miRNAs are predicted to target both KIT and ETV1, which are key factors in GIST oncogenesis. Therefore, the loss of expression of these miRNAs in GIST could be of importance for the molecular pathogenesis of these tumours, which makes them meaningful candidates for further research.

Inhibition of cell proliferation in miR-17, miR-20a and miR-222 overexpressing cells. Three miRNAs, miR-17, miR-20a and miR-222, were selected for functional characterisation, as these were the miRNAs with the highest expression fold change between GI-LMS and GIST compared with their cluster members. The downregulation of these miRNAs in GIST *vs* GI-LMS was validated by qPCR (Figure 2B, left panel). In addition, these miRNAs were downregulated in GIST compared with normal gastrointestinal tissues (Figure 2B, right panel). In contrast, in other tumour types, for example, colorectal cancer and non-small cell lung cancer, these miRNAs were upregulated or unaltered (Supplementary Figure 2). miRNA mimics and a scrambled control mimic were transfected into two well-characterised GIST cell lines, that is, GIST-T1 and GIST-882. Cell proliferation was monitored using SRB assays at 24 h, 48 h, 72 h and 96 h after transfection (Figure 3). In GIST-882 cells, miR-17 and miR-20a induced a strong inhibition of cell proliferation compared with the negative control, whereas miR-222 only slightly reduced cell proliferation within the time frame of the experiment. In GIST-T1 cells, miR-17, miR-20a and miR-222 overexpression caused a total proliferation arrest. These miRNAs also inhibited cell proliferation in GIST-T1-R cells that exhibit

Table 1. Patients' and tumours' characteristics

GIST	***n*=50**						
Gender		**Mutation status**		**Location**		**Risk of relapse[a]**	
Male	32	KIT		Gastric	36	Overtly malignant[b]	12
Female	18	*Exon 9*	4	Non-gastric:		High	12
		Exon 11	26	*Colon*	1	Intermediate	14
Median age (range)	65 (41–85)	*Exon 17*	1	*Duodenum*	2	Low	12
				Oesophagus	1		
		PDGFRA		*Small intestine*	10		
		Exon 12	1				
		Exon 14	1				
		Exon 18	17				
Leiomyosarcoma	***n*=10**						
Gender				**Location**		**Malignancy**	
Male	4			Gastric	7	Overtly malignant[b]	10
Female	6			Non-gastric			
				Small intestine	3		
Median age (range)	45 (36–71)						

[a]Tumour risk assessment was performed using AFIP criteria (Miettinen and Lasota, 2006).
[b]Recurrent or metastatic disease during clinical follow-up.

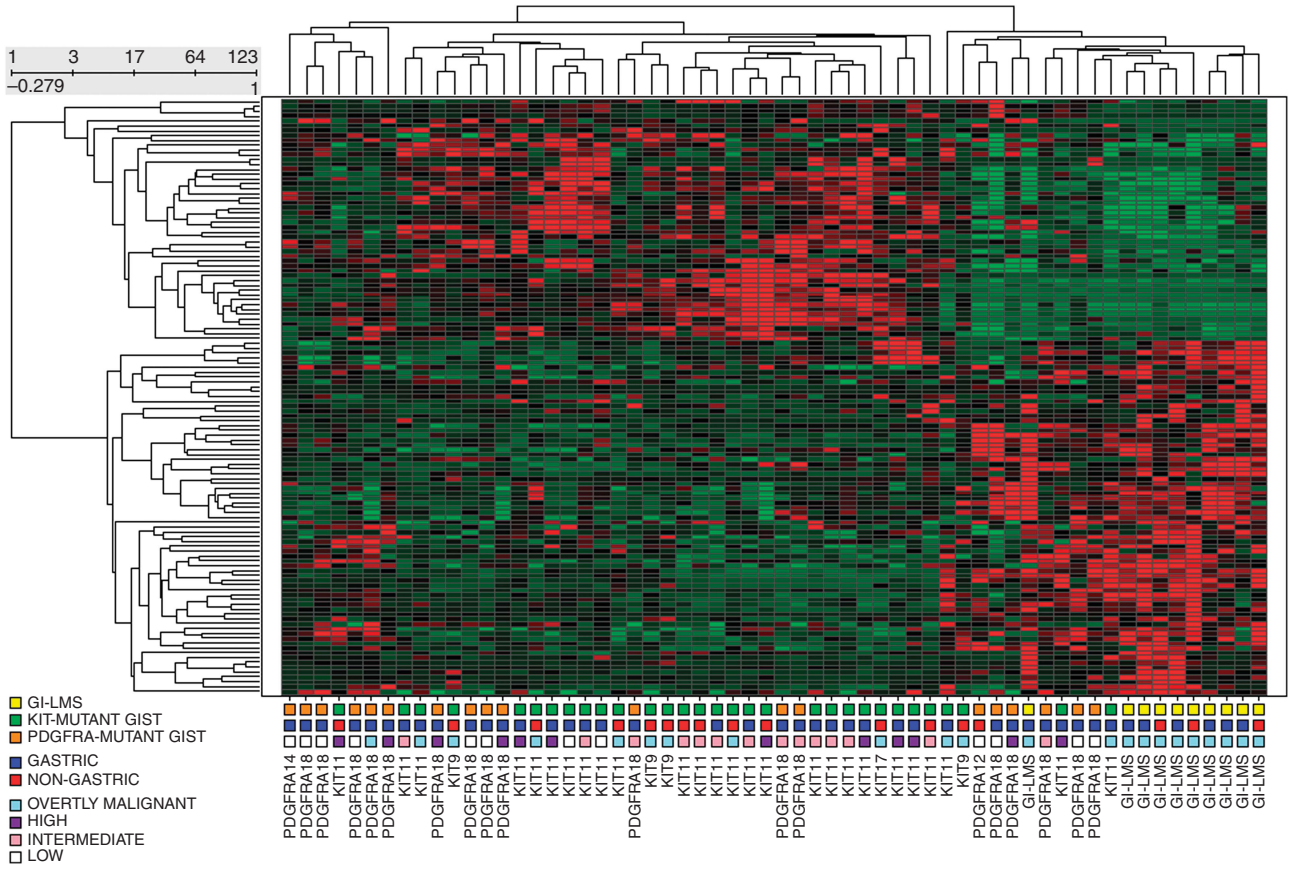

Figure 1. miRNA expression profiling clearly distinguishes the majority of GIST samples from GI-LMS. The most significant differentially expressed miRNAs (two-sample t-test, $P<0.005$, corrected for multiple testing $P<0.03$) between GI-LMS and GIST (either *KIT* or *PDGFRA* mutants) are used for a supervised hierarchical clustering. Tumour location (gastric *vs* non-gastric) and risk of relapse/malignancy (low/intermediate/high/overtly malignant) are indicated, as well as the *KIT/PDGFRA* exon that is mutated. The cluster tree was generated using the cosine correlation for similarity measures and UPGMA clustering method.

resistance to imatinib because of secondary *KIT* mutations (Supplementary Figure 3).

Overexpression of miR-17, miR-20a and miR-222 affects the cell cycle and induces apoptosis in GIST cells. Cell cycle profiles were determined 72 h post transfection of the miR-17, miR-20a and miR-222 mimics and scrambled control mimic (Figure 4A). GIST-T1 cells showed a small but consistent decrease in the percentage of cells in G1 and G2-M when miR-17 or miR-20a were overexpressed. At the same time, there was an increased sub-G1 fraction, indicative of cell death. A different effect was seen when miR-222 was overexpressed: there were just slightly more cells in the sub-G1 fraction, whereas there was a considerable decrease in cells in G1-phase. In addition, we observed an increased G2-M fraction indicating that cycling cells arrest in the G2-M phase when miR-222 is overexpressed.

In GIST-882 cells, similar observations were made, albeit less pronounced. As these cells proliferate less vigorously than GIST-T1 cells, the G1 peak is relatively high, which influences the cell cycle profile drastically. Nevertheless, there was still an increased sub-G1 fraction and a decreased G1 fraction in miR-17 and miR-20a overexpressing cells. In miR-222 overexpressing cells, there was a subtle decrease in the G1 peak, accompanied by an increased cell fraction in S-G2-M phase.

To determine the effect of miR-17, miR-20a and miR-222 overexpression on cell viability and apoptosis, GIST cells were transfected with miRNA mimics and a scrambled control mimic. Apoptosis was measured by Annexin V and PI staining determined by FACS-analysis 72 h post transfection (Figure 4B, for FACS plots see Supplementary Figure 4). In GIST-T1 cells, overexpression of the miRNAs led to a decreased population of live cells (Ann − / PI −) compared with the negative control. The early (Ann − /PI −) and late apoptotic fraction (Ann + /PI +) increased in the miRNA mimic-expressing cells. These results were similar for the GIST-882 cell line, although again the effects were less pronounced. However, regardless of the increased dead cell fraction (Ann − /PI +), miR-222 overexpression did not lead to increased levels of early and late apoptotic cells in this cell line, consistent with the results from the cell proliferation and cell cycle assays.

MiR-17, miR-20a target *ETV1* and miR-222 targets *KIT*. Candidate target genes for miR-17, miR-20a and miR-222 were predicted using the TargetScanHuman 6.2 (http://www.targetscan.org/) (Lewis *et al*, 2005). Interestingly, as mentioned, all miRNAs have potential binding sites in the 3′-UTR of *KIT* and *ETV1*, which are important factors in GIST oncogenesis (Figure 5). Indeed, *KIT* and *ETV1* mRNA expression were significantly higher in GIST compared with GI-LMS (Supplementary Figure 5).

MiRNAs can exert their regulatory function *via* mRNA degradation, translational repression or both. To determine the effects of miR-17, miR-20a and miR-222 overexpression on KIT and ETV1, both mRNA and protein levels were examined 72 h after transfection of GIST cell lines with the miRNA mimics and a negative control. In GIST-T1 cells, *ETV1* mRNA levels were 21% downregulated when miR-17 or miR-20a ($P=0.01$ for miR-20a) was overexpressed (Figure 6A). Overexpression of miR-222 barely

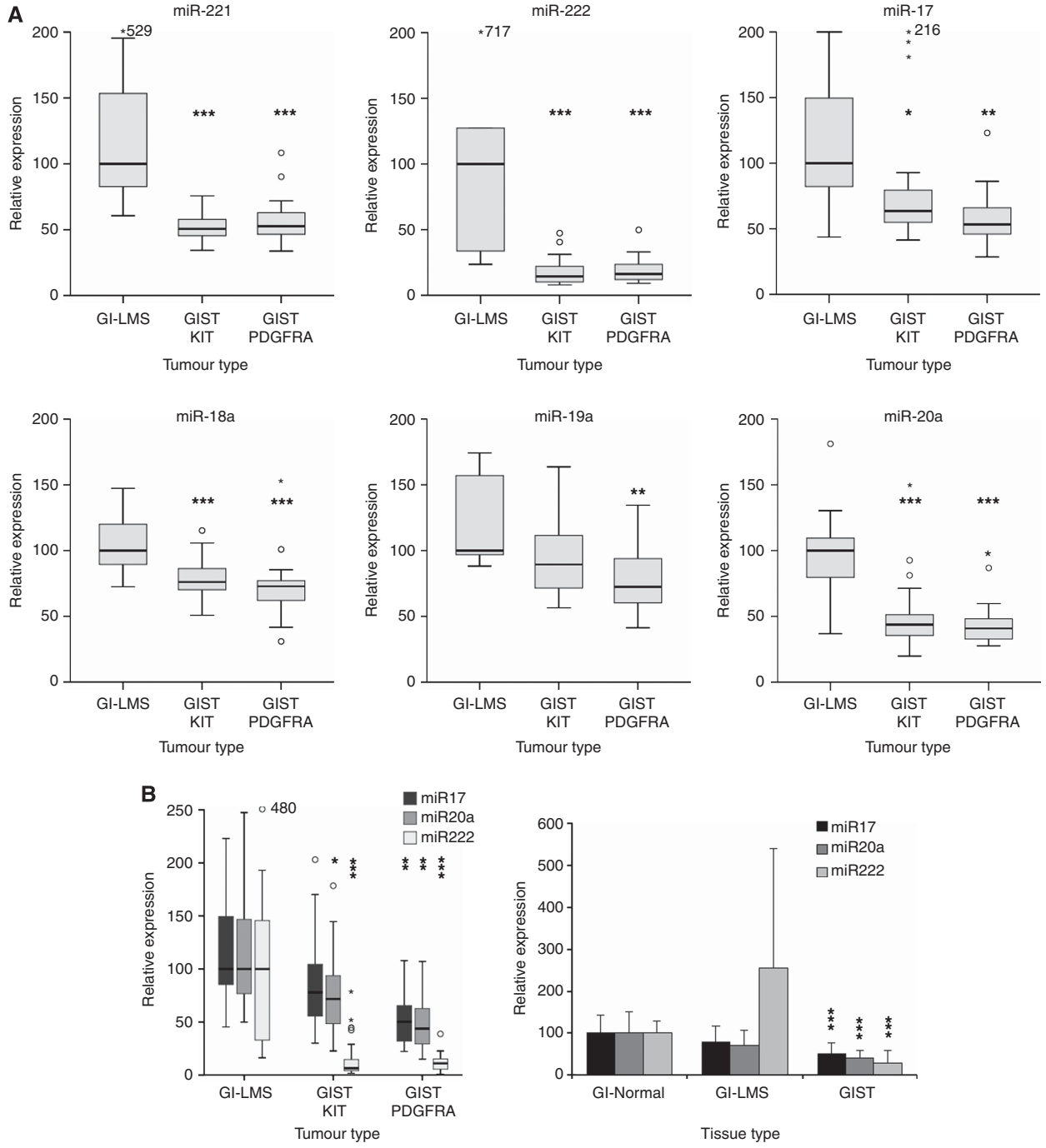

Figure 2. MiR-221/222 and miR-17-92 cluster members are downregulated in GIST compared with GI-LMS and normal GI tissues. (**A**) Boxplots represent miRNA microarray expression data of miR-221, miR-222, miR-17, miR-18a, miR-19a and miR-20a in GIST ($n=50$, either *KIT* or *PDGFRA* mutants) *vs* GI-LMS ($n=10$, median GI-LMS set at 100). (**B**) Left panel: MiR-17, miR-20a and miR-222 downregulation in GIST *vs* GI-LMS is validated by qPCR. Boxes represent 25th–75th percentile, line in the box represents median (50th percentile), whiskers represent the lowest and highest values within 1.5 × the interquartile range, circles are outliers (between 1.5-3x interquartile range), small asterisks are extreme values (>3x interquartile range). Numbers next to circles/asterisks correspond to values beyond scale. Significant differences in miRNA levels between GI-LMS and *KIT*- or *PDGFRA*-mutant GIST is determined using Mann Whitney *U*-tests. Right panel: miR-17, miR-20a and miR-222 are significantly downregulated in GIST compared with normal gastrointestinal tissues (GI-normal). miRNA expression levels of GIST/GI-LMS are relative to normal gastric ($n=5$) and intestinal tissues ($n=5$) (average GI-normal set at 100). Significant differences in miRNA levels between GIST ($n=50$) or GI-LMS ($n=10$) and GI-normal are determined using student *t*-tests. *$P<0.05$, **$P<0.005$, ***$P<0.0005$.

changed *ETV1* mRNA levels. *KIT* mRNA levels were hardly affected by miR-17, but significantly downregulated by miR-20a (19% decrease, $P=0.04$) and miR-222 (38% decrease, $P=0.02$). In GIST-882 cells, *ETV1* expression was not downregulated significantly upon mimic transfection, but *KIT* mRNA levels were reduced by 39% in miR-17- ($P=0.02$), 40% in miR-20a- ($P=0.02$) and 46% in miR-222 ($P=0.01$)-overexpressing cells. Protein expressions of both KIT and ETV1 were easily detectable by immunoblotting in both untransfected GIST cell lines and cells transfected with a scrambled control mimic (Figure 6B). However, cells overexpressing miR-17, miR-20a or miR-222 clearly showed diminished KIT and ETV1 protein levels in both cell lines.

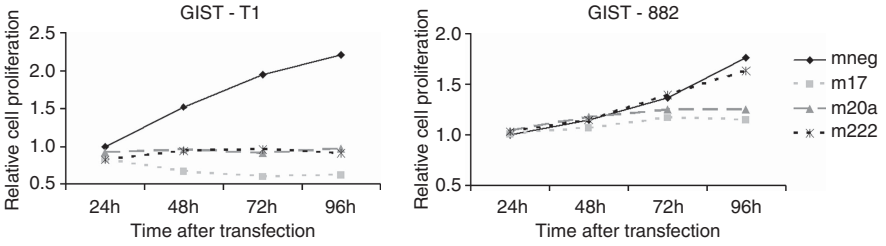

Figure 3. Reduced cell proliferation of GIST cell lines transfected with miR-17, miR-20a and miR-222 mimics. Graphs depict average cell density ($n=3$) of GIST-T1 and GIST-882 cell lines at 24 h, 48 h, 72 h and 96 h post transfection, relative to the negative control (mneg; transfected with scrambled mimic) at 24 h, which is set at 1.

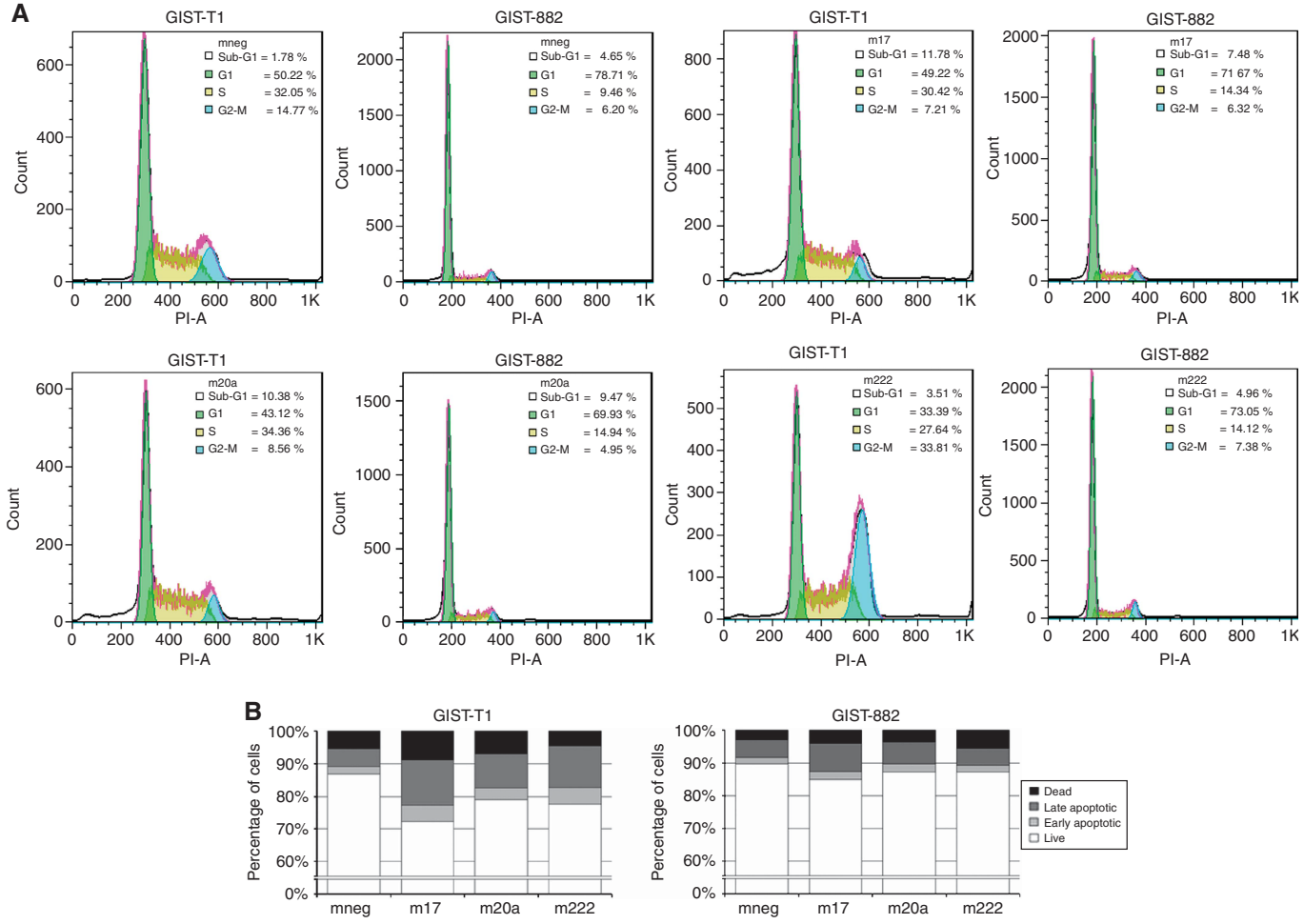

Figure 4. Overexpression of miR-17, miR-20a and miR-222 affects cell cycle progression and induces cell death. GIST-T1 and GIST-882 cell lines were transfected with scrambled (mneg), miR-17, miR-20a and miR-222 mimics. (**A**) Cell cycle profiles were determined by FACS analyses 72 h post transfection and analysed using FlowJo software. Sub-G1 cells are depicted in white, cells in G1-phase in green, cells in S-phase in yellow and G2-M-phase cells in blue. Percentages of cells in distinct stages of the cell cycle are depicted on the right. (**B**) FACS analysis of Annexin V/PI (Ann/PI)-stained cells, 72 h post transfection. Live cells are presented by the Ann-/PI- fraction, early apoptotic cells by the Ann+/PI- fraction, late apoptotic cells by the Ann+/PI+ fraction and dead cells are detected in the Ann-/PI+ fraction.

To demonstrate that the observed downregulation of KIT and ETV1 is due to the binding of the miRNAs to their 3′-UTRs, fragments of the 3′-UTRs spanning the potential miRNA target sites were cloned in a luciferase reporter construct. GIST cells were transfected with these constructs and miRNA mimics (Figure 6C). GIST-T1 cells with the ETV1short construct, containing binding sites for miR-17 and miR-20a, showed a 50% decreased luciferase activity ($P<0.0005$) when these miRNAs were overexpressed compared with the negative control. There was, however, no decreased luciferase activity when miR-222 was overexpressed, which agrees with the absence of a miR-222-binding site in the ETV1short construct. When a potential miR-222-binding site was introduced, as is the case in the ETV1long construct, miR-222 overexpression led to a small but significant decrease (12%, $P=0.02$) in the luciferase activity.

Both KIT constructs comprise potential binding sites for miR-17/20a and miR-222. Using both constructs, overexpression of miR-17 and miR-20a resulted in a 17–30% decrease ($P<0.02$) in

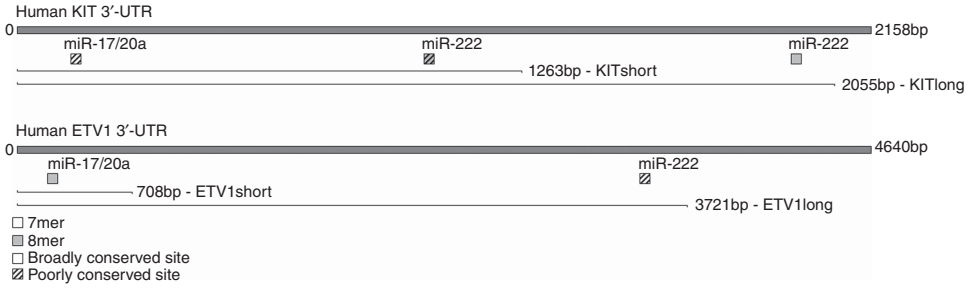

Figure 5. 3′-UTR of *KIT* and *ETV1* and predicted target sites (TargetScanHuman 6.2) of miR-17, miR-20a and miR-222. Lines indicate the 3′-UTRs fragments (KITshort/long, ETV1short/long) that are cloned into the psiCHECK-2 luciferase reporter.

luciferase activity, whereas miR-222 showed a stronger inhibition of luciferase activity (39%–58% decrease, $P<0.002$). The presence of an additional distal miR-222 site in the KITlong construct did not further decrease luciferase activity. For all 3′-UTR constructs, a combination of miR-17, miR-20a and miR-222 mimic (mix) did not have a synergistic effect on the reduction of luciferase activity compared with the overexpression of individual miRNAs.

To examine whether the regulation of KIT and ETV1 is due to direct miRNA binding to target sites in the 3′-UTRs, the putative binding sites were mutated in the ETV1short, ETV1long and KITshort constructs (Figure 6D, Supplementary Figure 1). GIST-T1 cells transfected with the ETV1short construct containing a mutated miR17/20a site, showed a significantly higher luciferase activity when transfected with miR-17 (84% *vs* 52%, $P<0.0005$) and miR-20a (77% *vs* 47%, $P<0.0005$) compared with cells transfected with the wild-type ETV1short construct. Similarly, in ETV1long, mutation of the miR-17/20a target site significantly restored luciferase activity (miR-17: $P=0.03$; miR-20a: $P=0.002$). It was noted that luciferase activities were never restored to the levels obtained in cells transfected with the mneg control mimic. This may be caused by the severe effects overexpression of the miR-17/20a mimics have on GIST-T1 cell proliferation and survival. Apparently miR-222 does not directly regulate ETV1, as mutation of the miR-222-binding site did not lead to increased luciferase activity. Similarly, it can not be firmly concluded that miR-17 and miR-20a directly regulate KIT. In contrast, mutation of the miR-222-binding site in the KIT 3′-UTR did significantly abolish regulation by miR-222 (72% *vs* 49%, $P=0.01$).

As the reduction in luciferase activity upon miRNA overexpression is more prominent than the downregulation of KIT and ETV1 mRNA levels, the miRNA-mediated inhibition of these genes apparently takes place *via* translational repression rather than *via* mRNA degradation. The marked decrease in KIT and ETV1 protein levels upon miR-17, miR-20a and miR-222 overexpression in GIST cell lines supports this theory.

DISCUSSION

Deregulation of miRNA expression can contribute to tumorigenesis, and often the deregulated miRNA expression patterns are tumour type specific. In order to identify miRNAs that are specifically deregulated in GIST, miRNA expression was compared with that of GI-LMS, another mesenchymal tumour type with similar morphological features that occurs at the same anatomical sites. Here we showed that there is a large disparity in miRNA expression between GIST and GI-LMS.

The expression levels of the miR-221/222 and miR-17-92 clusters were significantly lower in GIST compared with GI-LMS. This was confirmed by qPCR, which also showed that the expression of miR-17, miR-20a and miR-222 was lower in GIST than in normal GI tissues. In order to explain the reduced levels of these miRNAs in GIST, we checked whether chromosome 13 loss, which is frequently observed in advanced GIST, is correlated to lowered expression of miR-17 and miR-20a of which the genes are located on 13-q31.3. In our sample set no such correlation could be observed. An alternative explanation may be epigenetic silencing that has been reported for the miR-17-92 cluster in the macrophages and lung tissue (Pospisil *et al*, 2011; Dakhlallah *et al*, 2013). Interestingly, members of the miR-17-92 cluster are often found higher expressed in human malignancies, including gastric and colon cancers, neuroblastoma and B-cell lymphomas relative to normal tissue (He *et al*, 2005; Volinia *et al*, 2006; Guo *et al*, 2009; Schulte *et al*, 2010). Also the miR-221/222 cluster is known to be upregulated in various cancers such as glioblastoma, and hepatocellular, pancreatic and prostate carcinoma (Galardi *et al*, 2007; Gillies and Lorimer, 2007; Lee *et al*, 2007; Fornari *et al*, 2008). In line with our data, it was previously reported that the expressions of miR-221 and miR-222 are significantly downregulated in GIST in comparison with the peripheral non-tumourous tissue (Koelz *et al*, 2011). miRNAs belonging to both clusters have been considered to act as either a tumour suppressor or an oncogene depending on the tumour type (O'Donnell *et al*, 2005; Garofalo *et al*, 2012).

Being important oncomiRs, the relatively low expression of the miR-221/222 and miR-17-92 cluster members could contribute to GIST development. In support of this notion, increased expression of miR-17, miR-20a and miR-222 in two different, well-characterised GIST cell lines resulted in cell growth inhibition and apoptosis induction. In addition, cells overexpressing miR-222 showed an elevated G2-M peak. This could be the result of a mitotic block, or a forced entrance to G2/M while the cells are not ready to undergo mitosis, which subsequently leads to cell death.

MiR-221/222 is reported to inhibit cell proliferation *via* KIT down-modulation in erythropoiesis and erythroleukemic cells (Felli *et al*, 2005). In GIST, downregulation of miR-221 and miR-222 is correlated with pronounced KIT expression (Koelz *et al*, 2011). However, in that study, functional experiments to investigate the cellular effects of miR-221/222 repletion in GIST cells were not performed. MiR-494 has been demonstrated to target and downregulate KIT thereby inhibiting GIST cell growth and inducing apoptosis (Kim *et al*, 2011). Interestingly, target prediction algorithms not only identified potential miRNA-binding sites in the *KIT*-3′-UTR for miR-221/222, but also for miR-17-92 cluster members. The upregulation of miR-17, miR-20a or miR-222 in our GIST cell lines resulted in downregulation of *KIT* mRNA and markedly reduced protein levels. Luciferase reporter assays showed clear regulation of a *KIT*-3′-UTR controlled luciferase signal by miR-222, confirming that miR-222 directly targets *KIT*. As we could not demonstrate direct regulation of *KIT* by miR-17/20a, we consider the observed reduction of KIT protein in GIST cells transfected with miR-17/20a to be an indirect secondary effect, possibly mediated through downregulation of additional target genes.

Recently ETV1 was found to be highly expressed in GIST and required for GIST development. We observed that the upregulation of miR-17, miR-20a or miR-222 not only affected KIT expression but also resulted in the downregulation of ETV1 protein levels in two GIST cell lines. We show that miR-17 and miR-20a directly target *ETV1*, whereas miR-222 does not seem to regulate ETV1 in a direct manner. As it is known that mutated KIT prolongs ETV1 stability (Chi *et al*, 2010), it may be that the lowered KIT levels caused by miR-222 affect the stability – and hence protein levels – of ETV1. Alternatively, KIT-independent factors, possibly other miR-222 target genes, could be responsible for the reduced ETV1 protein levels.

In addition to miR-17 and miR-20a, other members of the miR-17-92 cluster, which were also downregulated in GIST, have potential target sites in *KIT* and *ETV1*. The 3′-UTR of *KIT* contains putative target sites for miR-18a (and the closely related miR-18b from another cluster, but belonging to the miR-17 family) and miR-19a/b, whereas the *ETV1*-3′-UTR contains a potential miR-19a/b site. Future research will demonstrate whether these miRNAs are capable of regulating KIT and ETV1 levels *via* these putative target sites. It was also noted that the 3′-UTR of *PDGFRA* contains two potential miR-17/20a target sites. Therefore, overexpression of miR-17 and miR-20a potentially also has an effect on *PDGFRA*-mutant GIST.

Although miR-17 and miR-20a regulate ETV1 and miR-222 regulates KIT, they will most likely also target additional genes in the context of GIST cells. The cumulative effect of all target genes will eventually explain the cellular phenotype observed, including the differences between the miR-17/20a and miR-222 effects.

As GIST cells are dependent on the continued high expression of KIT and ETV1, miRNAs that target these genes present a promising therapeutic opportunity. Despite the enormous successes obtained with KIT-targeting tyrosine kinase inhibitors (TKIs) such as imatinib and sunitinib, the vast majority of patients with metastatic GIST will eventually become resistant against these drugs, for example, due to secondary mutations in the tyrosine kinase receptors. Moreover, the majority of WT-GIST and *PDGFRA*-D842V-mutant GIST are not sensitive to imatinib in the first place. Therefore, inhibition of KIT activity alone is unlikely to cure GIST (Rubin, 2010). We confirm miR-222 as a regulator of KIT, and are the first to demonstrate that miR-17 and miR-20a regulate ETV1, another key factor in GIST development. Moreover, we present evidence that overexpression of these miRNAs in GIST cell lines dramatically inhibits cell proliferation, affects cell cycle progression and induces apoptosis. Remarkably, our results demonstrate that miR-17/20a clearly affect, in an indirect manner, KIT expression; similarly, miR-222 has a pronounced effect on ETV1 expression. As of yet, it is unclear which genes and molecular mechanisms underlie these observations. Although miRNA therapy is still in its infancy and further *in vivo* research needs to be done, our findings could hold great therapeutic potential (Cho, 2010; Kasinski and Slack, 2011; Thorsen *et al*, 2012). When miRNA treatment becomes available, targeted overexpression of members of the miR-221/222 and miR-17-92 clusters in GIST might significantly suppress ETV1 and KIT levels. The joined repression of both essential GIST oncogenes could be promising for GIST treatment, especially for imatinib-resistant disease.

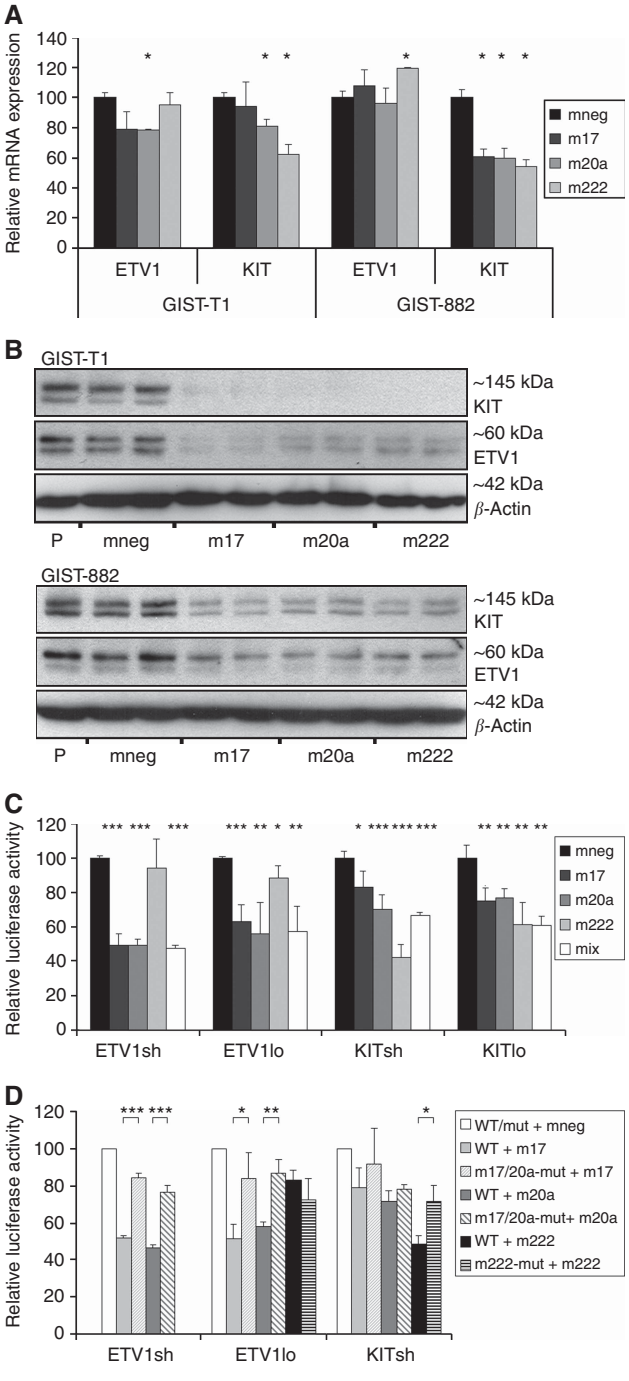

Figure 6. MiR-17, miR-20a and miR-222 target the 3′-UTR of *KIT* and *ETV1*, and thereby reduce their mRNA and protein levels. GIST-T1 and GIST-882 cell lines were transfected with scrambled (mneg), miR-17, miR-20a and miR-222 mimics and protein, and RNA was isolated 72 h post transfection. (**A**) *KIT* and *ETV1* mRNA expression were determined by qPCR and normalised to three housekeepers (*GAPDH*, *HPRT* and *HMBS*). Shown are average values ± s.d. ($n=2$), average expression of mneg is set at 100. (**B**) KIT and ETV1 protein expression were determined by SDS–PAGE and immunoblotting. Parental untransfected control GIST cell lines are indicated by P. β-Actin was used as loading control. (**C**) GIST-T1 cells were transfected with psiCHECK-2 constructs containing short (sh) and long (lo) fragments of 3′-UTR sequences of *ETV1* and *KIT* ($n=4$), followed 24 h later by transfection of scrambled (mneg), miR-17, miR-20a or miR-222 mimics, or a mixture of these miRNA mimics (mix). (**D**) GIST-T1 cells were transfected with psiCHECK-2 constructs containing wild-type (WT) and mutated (mut) 3′-UTR sequences of ETVshort (sh), ETVlong (lo) and KITshort, followed 24 h later by transfection of scrambled (mneg), miR-17, miR-20a or miR-222 mimics. The *Renilla* luciferase activity was measured and normalised using firefly luciferase activity 48 h after final transfection. The relative luciferase activity of cells transfected with scrambled mimic was set at 100. Shown are average values ± s.d. ($n=3$). Statistical significance between scrambled control and miRNA mimics or between WT and mut was tested by two-sample *t*-test. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.0005$.

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