An Investigation of miRNAs in the Pathogenesis of Paediatric/Wild-Type Gastrointestinal Stromal Tumour

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Introduction

Gastrointestinal Stromal Tumors (GISTs) may arise at any age but most commonly occur in men aged 55-65 years[1] and are characterized by activating mutations of tyrosine kinase III receptors KIT or PDGFRα. These mutations are mutually exclusive and are the initiating oncogenic event in GIST development. Around 10-15% GISTs contain no detectable KIT or PDGFRα mutations, however a small percentage bear a B-RAF mutation. The remainder are known as wild-type (WT) GISTs. Pediatric GISTs are a subset of wild-type GISTs. They are almost always KIT immunopositive and have similar downstream signalling to adult mutant tumors, yet lack the activating mutations. They are generally slow-growing tumors, predominantly occur in prepubescent girls in a gastric location and show epitheloid morphology.

While adult GISTs show large-scale genomic losses of chromosomes 14q, 22q, 1p and 9p with progression, these changes are not seen in the pediatric setting. Different mRNA expression profiles have also been identified in adult and pediatric GISTs. Strong IGF1R over-expression has been found in pediatric GIST with no detectable mutations or gene amplification to explain this[2]. IGF1R over-expression is important as a potential therapeutic target, currently being assessed by clinical trial for pediatric GISTs.

WT GISTs can be associated with a number of syndromes: Neurofibromatosis, Carney's Triad and Carney-Stratakis Syndrome [dyad]. Carney's Triad is the association of GIST with extra-adrenal paragangliomas and pulmonary chrondromas. Carney-Stratakis Syndrome is the association of familial paraganglioma with GIST. The dyad is distinct from Carney's Triad in that it is an inherited syndrome, caused by mutations in the succinate dehydrogenase (SDH) subunits B, C or D[3]. Inactivation of any one SDH subunit leads to enzyme complex destabilization and loss of function. Absent SDHB expression has been reported in pediatric (WT) GISTs[4] and in GISTs of Carney's Triad[5], with SDH mutations found in only 12% of cases[4]. By contrast, SDHB is strongly expressed in KIT- and PDGFRα-mutant GISTs.

Given that many of the key differences between adult and pediatric GIST are not readily explained on a genetic level, we hypothesize that pediatric GIST is driven by epigenetic dysregualtion, specifically microRNAs (miRNAs) and aberrant methylation.

MicroRNAs are single stranded, non-coding RNAs typically 19-25 nucleotides in length. They are negative regulators of gene expression and control a wide range of biological functions, including proliferation and apoptosis. Aberrantly expressed miRNAs have been implicated in many cancers. miRNA profiling of adult mutant GISTs showed differential expression of 32 miRNAs based on anatomic tumour location and mutational status, and differential expression of 44 miRNAs clustered on 14q32.31 corresponding to 14q genomic loss[6]. Another study found miRNA expression patterns were related to 14q loss, anatomic location and risk, but not KIT or PDGFRα mutation status[7]. While these studies provided some insights into adult GIST, they did not include any pediatric (WT) cases.

The aim of this study is to investigate the role of the epigenetic mechanisms in GIST biogenesis in the pediatric setting.

Methodolgy

Sample Cohort

Samples were collected from European and US pathology and oncology colleagues. Age categorization was: <25 years = pediatric and >/=25years = adult. Mutational status was based on analysis conducted in the laboratory of MD-R and MOS. Our final cohort included 30 adult mutant, 15 adult WT and 29 pediatric WT cases (table 1), with a male: female ratio of 1.4:1 in adult mutant, 1:3 in adult WT and 1:2.6 in pediatric WT cases.

Mutational Testing

Tumour DNA was PCR amplified for KIT exons 9, 11, 13, 17 and PDGFRA exons 12, 14, and 18 using well established primers (M D-R). The products were examined by High Resolution Melt Analysis and those with aberrant melt curves were subjected to sequencing.

MicroRNA Profiling

Formalin Fixed Paraffin Embedded (FFPE) tumour samples were micro-dissected for tumour only and RNA was extracted using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion® Austin TX). Reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and MegaPlex™ RT primers (Applied Biosystems) pool A or pool B, which allowed the reverse transcription of 381 miRNAs in one pool. MegaPlex™ RT products were pre-amplified using TaqMan® PreAmp MasterMix and PreAmp primers (pool A or B) (Applied Biosystems). MicroRNA profiling was performed with TaqMan® Low Density Arrays (TLDAs)(Applied Biosystems)for a total of 667 miRNAs using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Statistical Analysis

Ct values over 35 were considered noise and were disregarded. miRNAs not expressed in greater than 20% of samples were also excluded from analysis. Mean normalization was carried out by subtracting the mean sample Ct from individual Ct values. Normalized relative expression (NRQ) or miRNA was calculated with reference to Ct max using: NRQ=2(Ctmax-Ct). Clustering, heatmap generation and boxplots were performed using hclust, heatmap.plus and boxplot packages from R statistical computing language v2.8.1. Hierarchical clustering was performed using Spearman's rank correlation and Ward's linkage. Heatmap colouring was based on rank of sample value across each miRNA. Statistical significance of change in miRNA expression level over various sample classes was calculated using Wilcoxon's rank-sum test and corrected for multiple comparisons using Bonferroni method. miRNA: gene interactions were investigated by assessing the number of observed interactions (listed in TargetScan) compared to those expected by chance using MirMatcher, a custom-built software application, implemented in Java.

TLDA Validation

Validation was performed by individual TaqMan® microRNA RT-qPCR assays for selected miRNAs. RNA was reverse transcribed using the TaqMan® microRNA reverse transcription Kit and reverse transcription primers specific for individual miRNAs (Applied Biosystems). qPCR was performed with microRNA specific TaqMan® probes and TaqMan® Fast Universal PCR MasterMix (2X) No AmpErase® UNG (Applied Biosystems). Results were analysed using the RQ manager 1.2.1 software (Applied Biosystems).

	Adult Mutant	Adult WT	Pediatric WT
	n=30	n=15	n=29
Male	17 (59%)	4 (25%)	8 (28%)
Female	12 (41%)	12 (75%)	21 (72%)
Age, mean±SD	59.6±15.4	45.1±12.7	16.1±6
Location n (%)			
Stomach	29 (100% by design)	12 (75%)	27 (93%)
Jejunum	0	1 (6%)	2 (7%)
Ileum	0	2 (13%)	0
Retroperitoneum	0	1 (6%)	0
KIT Mutation n (%)	18 (63%)	WT	WT
PDGFRα Mutation n (%)	11 (38%)	WT	WT
14q Status n (%)			
Loss	23 (85%)	NA	NA
Diploid	3 (11%)	NA	NA
Triploid	1 (4%)	NA	NA

Table 1
Sample demographics

Results

miRNA Profiling

Unsupervised hierarchical clustering revealed a clear separation between adult mutant and pediatric WT GISTs. The dendrogram revealed a further clear split within the adult mutant cohort, due to differential expression of forty-eight miRNAs located on chromosome 14q32.2 and 14q32.31 (figure 1). This is not simply explained by genomic loss of 14q, as the majority of cases in fact show 14q32 loss by fluorescence in situ hybridization analysis [FISH]. Interestingly, the pediatric small bowel GISTs cluster with the adult mutant cases, while the adult WT cases are dispersed amongst adult mutant and pediatric cases.

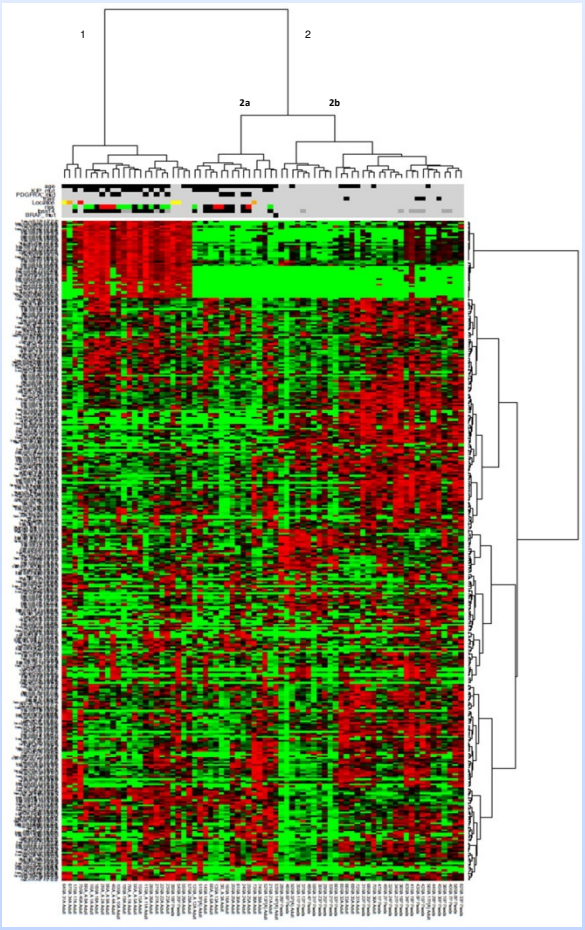


Figure 1
Unsupervised hierarchical clustering splits the adult samples into 2 clusters: 1 and 2a. This is caused by high expression of 48 miRNAs located on 14q32.2 and14q32.31 (cluster 1). Pediatric WT small bowel samples cluster with these also.

The data were interrogated for miRNAs which A) showed differential expression >5-fold, B) target genes known to be important in GIST biology and C) were of statistical significance (p<0.05) between the groups: 1) adult mutant vs. pediatric WT, 2) adult WT vs. pediatric WT, 3) adult mutant vs. adult WT, 4) KIT vs. PDGFRα and 5) high- vs. low-risk adult mutant. Results for analyses 1 and 2 revealed that the number of interactions between miRNAs and gene targets are significantly greater than expected (p<0.0007 and p<0.02 respectively).

The data were then investigated for potential biological interactions between diametrically expressed miRNA and mRNA [from published expression data] for the comparisons: 1) Genes higher in pediatric compared to adult mutant – miRNAs lower in pediatric compared to adult mutant, 2) Genes lower in pediatric compared to adult – miRNAs higher in pediatric compared to adult, 3) Genes higher in mutant compared to WT – miRNAs lower in mutant compared to WT, 4) Genes higher in WT compared to mutant – miRNAs lower in WT compared to mutant, 5) Genes higher in pediatric compared to adult WT – miRNAs lower in pediatric compared to adult WT and 6) Genes higher in pediatric compared to adult mutant – miRNAs lower in pediatric compared to adult mutant. Comparisons 1 and 6 (tables 2A &2B) were found to have a significant number of interactions, p<0.0122 and p<0.006 respectively, more than expected for the set of miRNAs and randomly selected genes. Comparison 4 (table 2C) was found to be significant (p<0.013) when only conserved targets were examined.

Comparison 1	
Genes	**miRNAs**
IGF1R	hsa-let-7b
NLGN4	hsa-let-7f
ANK3	hsa-139-5p
FZD2	hsa-miR-340
PHKA1	hsa-miR-455-5p
	hsa-miR-152
	hsa-miR-193b
	hsa-miR-365
	hsa-miR-484
	hsa-miR-886-3p
	hsa-miR-886-5p

A Table 2 A) Diametrically expressed miRNA and mRNA for the comparison genes relatively over-expressed in pediatric versus adult mutant cases

Comparison 6	
Genes	**miRNAs**
IGF1R	hsa-let-7b
ANK3	hsa-let-7f
FZD2	hsa-139-5p
	hsa-miR-340
	hsa-miR-455-5p
	hsa-miR-152
	hsa-miR-193b
	hsa-miR-365
	hsa-miR-886-5p

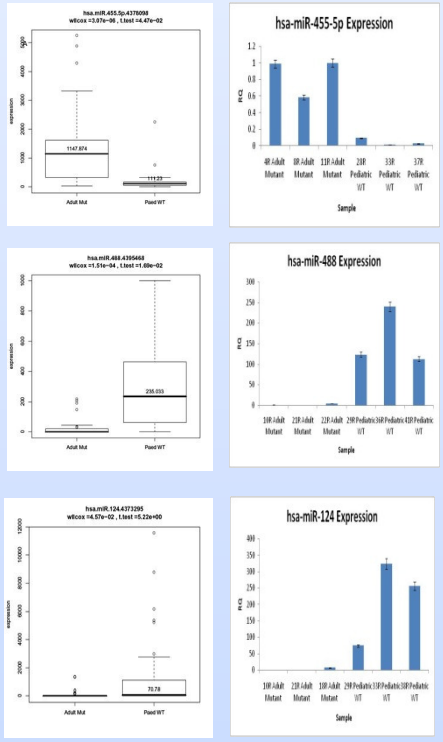
B Table 2 B) Diametrically expressed miRNA and mRNA for the comparison genes relatively over-expressed in pediatric WT versus adult mutant cases

Comparison 4	
Genes	**miRNAs**
VEGFA	hsa-miR-302b
BCL2	hsa-miR-410
GLUT1	
IL2	

Table 2 C) Diametrically expressed miRNA and mRNA for the comparison genes relatively over-

Validation

Three miRNAs were selected for validation with individual TaqMan ®microRNA assays. These were selected based on the above criteria: >5-fold difference, target genes of interest and statistically significant. The miRNAs chosen for validation were hsa-miR-455-5p, hsa-miR-488 and hsa-miR-124.



A B
Figure 2
A)hsa-miR-455-5p, 488 & 124 boxplots from comparisons.
B)RT-qPCR validation results

Findings

- Cluster Analysis shows separation of adult mutant from paediatric wild-type GIST
- The Adult mutant cases are split according to expression of miRNAs on 14q. This split is NOT purely based on genomic 14qloss
- Adult WT GISTs mainly cluster with paediatric WT cases
- Small bowel WT cases cluster with adult mutant GISTs
- Several statistically significant differentially expressed miRNAs are predicted to target genes of known importance in GIST biology, notably IGF1R, SDH and VEGF

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