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An miRNA Signature Predicts Grading of Pancreatic Neuroendocrine Neoplasms

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Abstract. *Background/Aim: Grading pancreatic neuroendocrine neoplasms (PNENs) via mitotic rate and Ki-67 index score is complicated by interobserver variability. Differentially expressed miRNAs (DEMs) are useful for predicting tumour progression and may be useful for grading. Patients and Methods: Twelve PNENs were selected. Four patients had grade (G) 1 pancreatic neuroendocrine tumours (PNETs); 4 had G2 PNETs; and 4 had G3 PNENs (2 PNETs and 2 pancreatic neuroendocrine carcinomas). Samples were profiled using the miRNA NanoString Assay. Results: There were 6 statistically significant DEMs between different grades of PNENs. MiR1285-5p was the sole miRNA differentially expressed (p=0.03) between G1 and G2 PNETs. Six statistically significant DEMs (miR135a-5p, miR200a-3p, miR3151-5p, miR-345-5p, miR548d-5p and miR9-5p) (p<0.05) were identified between G1 PNETs and G3 PNENs. Finally, 5 DEMs (miR155-5p, miR15b-5p, miR222-3p, miR548d-5p and miR9-5p) (p<0.05) were identified between G2 PNETs and G3 PNENs. Conclusion: The identified miRNA candidates are concordant with their patterns of dysregulation in other tumour types. The reliability of these DEMs as discriminators of PNEN grades support further investigations using larger patient populations.*

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The most recent grading system for pancreatic neuroendocrine neoplasms (PNENs) was informed by the 2017 WHO classification, which uses quantification of the Ki-67 proliferative index and mitotic rate as the 2 principal metrics for grading pancreatic neuroendocrine tumours (PNETs). This updated classification further refines grade (G) 3 as a category that is distinctly comprised of either well differentiated neuroendocrine tumours (NETs) or poorly differentiated neuroendocrine carcinomas (1-4). This clarification of G3 is corroborated by studies that used next-generation sequencing to demonstrate that there are distinct genomic alterations that distinguish well differentiated G3 NETs from poorly differentiated G3 neuroendocrine carcinomas (5-7).

Given the interobserver variability in determining Ki-67 proliferative index scores and mitotic rates, reliance upon these metrics could potentially complicate the assignment of a PNEN's grade, especially for cases with a Ki-67 proliferative index score or mitotic rate that is at the borderline between grades. Moreover, mimickers, which can include apoptotic bodies, lymphocytes, and darkly stained nuclei, can confound determination of true mitotic figures and/or Ki-67–positive tumour cells and can further complicate cases that do not have a clear-cut grade (8).

Understandably, recent investigative efforts have evaluated ancillary methods to assist in improving the accuracy of grade assignment and interobserver reproducibility. For example, phosphorylated histone H3 (pHH3) has been used as a mitotic marker for mitotic rate assessment, and double-staining for Ki-67 with CD45 has been used to exclude lymphocytes as mimickers (9-15).

Studies have shown the utility of a profile or signature of microRNAs (miRNAs) and demonstrate that differentially expressed miRNAs (DEMs) can be used to distinguish between lower and higher grades of cancer (16-18). There have also been previous studies focused on using DEMs to characterize

NETs (19-24). For instance, an miRNA expression profile identified significant DEMs that can differentiate types of neuroendocrine lung neoplasms (including typical carcinoids, atypical carcinoids, large-cell neuroendocrine lung cancer and small-cell lung cancer) (20). DEMs have been identified as prognostic predictors that effectively distinguish lower-grade (G1) and higher-grade (G2/3) NETs of the small intestine (19, 25). There have also been preliminary studies comparing DEMs from PNETs to DEMs from background pancreatic islets, as well as studies focused on determining candidate DEMs with prognostic value (26-28).

Given the precedent of previous studies that have used the NanoString platform to reliably identify DEMs for different tumour types (29, 30), we used the NanoString assay to identify DEMs that may be useful in differentiating PNETs by grade.

Patients and Methods

Patient selection. This retrospective study was approved by the Moffitt Cancer Center Institutional Review Board (IRB). Twelve patients with PNENs of different grades were selected from the pathology repository at Moffitt Cancer Center. A retrospective chart review was performed using PowerChart/PathNet.

Pathologic evaluation of samples. The relevant haematoxylin and eosin– (H&E-) stained slides for all selected cases underwent a comprehensive histopathologic review by 2 pathologists (DC and JS) to confirm their diagnoses. To achieve the highest possible tissue purity for each specimen, we excluded (*via* macrodissection) the other tissue components on the slide/block before the sample was submitted for NanoString analyses. The tissue collection and review processes were regulated by specific standard operating procedures and quality assurance/quality control (QA/QC) protocols. We juxtaposed the marked glass slides to the corresponding paraffin block, and using a scalpel, we macrodissected the marked PNEN area. The samples were submitted to the Moffitt molecular pathology laboratory, where RNA was extracted and subjected to NanoString analysis, as described below.

NanoString. The samples were profiled using the miRNA NanoString Assay (NanoString Technologies, Seattle, WA, USA). The NanoString procedures were performed as previously described (31). In brief, 100 ng of RNA was extracted from each formalin-fixed paraffin-embedded (FFPE) block and used as input. Mature miRNAs were ligated to a species-specific tag sequence (miRtag) using a thermally controlled splinted ligation. Unligated miRtags were removed with enzymatic purification, and miR-tagged mature miRNAs were then hybridized with an nCounter Human (V2) miRNA Expression Assay CodeSet (NanoString Technologies) overnight at 65°C. The unhybridized CodeSet was removed with automated purification, which was performed on an nCounter Prep Station (NanoString Technologies), and the remaining target:probe complexes were transferred and bound to an imaging surface, as previously described. Counts of the reporter probes were tabulated for each sample by the nCounter Digital Analyzer (NanoString Technologies), and raw data output was imported into nSolver (http://www.nanostring.com/products/nSolver) (32). The nSolver procedures have been previously described (31); in brief, positive control probes in the CodeSet were tested for their linearity with a correlation between the concentration of the added target and the resulting count; correlation ≥0.95 indicated high-quality data. The limit of detection for each assay was confirmed using positive and negative controls; the positive control (Pos-E, 0.5fM) was above the average of the negative control means (NanoString Technologies I. nCounter Expression Data Analysis Guide) (33). Data was normalized based on housekeeping genes (B2M, GAPDH, RPL19, ACTB and RPLP0) and transformed into log2 scale.

Statistical analysis. The normalized miRNA data were analysed using SAS and R software. Wilcoxon rank sum test or Kruskal-Wallis test were performed on 800 measured miRNAs to identify the most commonly differentially expressed miRNAs between different grades of PNENs. Further analyses were performed to identify miRNAs that had statistically significant differential expression between specific grades of PNENs. Due to the small sample size (n=12), the analysis was for exploratory purposes and not intended for correction of multiple testing.

Results

Of all 12 patients with PNENs, 4 had G1 PNETs, 4 had G2 PNETs, and 4 had G3 PNENs, of which 2 were PNETs and 2 were pancreatic neuroendocrine carcinomas. Seven patients were male (58%), and the average patient age was 62.6 years (range=40-85 years). Eight PNENs involved the pancreatic tail, 2 involved the pancreatic head, 1 involved the body of the pancreas, and 1 was non-specified. The average PNEN tumour size was 3.89 cm (range=1.7-7.5 cm).

Of the 8 G1/2 PNETs, 1 G1 PNET was stage pT1, 3 were pT2, and 4 were pT3. All 4 G3 PNENs were stage pT3. Two patients with G2 PNETs had previously received neoadjuvant therapy, whereas the 10 remaining patients received no presurgical therapy. Two of the 8 G1/2 PNETs were metastatic, and 1 of the 4 G3 PNENs was metastatic. All 8 of the G1/2 PNETs had negative margins, and 2 of the 4 G3 PNENs had negative margins.

We identified miRNAs that had statistically significant differential expression between specific grades of PNENs; additionally, we identified miRNAs that had statistically significant differential expression consistently observed between all 3 grades of PNENs. Overall, there were 9 miRNAs (miR1285-5p, miR222-3p, miR200a-3p, miR3151-5p, miR15b-5p, miR155-5p, miR-345-5p, miR548d-5p and miR9-5p) that had a statistically significant differential expression (p<0.05) between different grades of PNENs. Of these, there were 6 statistically significant DEMs (miR1285-5p, miR15b-5p, miR155-5p, miR-345-5p, miR548d-5p, and miR9-5p) (p<0.05) between different grades of PNENs (G1/2/3) (Table I, Table II, Table III, and Table IV).

MiR1285-5p was the sole miRNA with a statistically significant differential expression (p=0.03) between G1 and G2 PNETs. Six statistically significant DEMs (miR135a-5p, miR200a-3p, miR3151-5p, miR-345-5p, miR548d-5p, and miR9-5p) (p<0.05) differentiated G1 PNETs from G3 PNENs

Table I. *Comparison of miRNAs with statistically significant differential expression between grade 1, 2 and 3 pancreatic neuroendocrine neoplasms (NENs).*

miRNA	Grade, mean (SD); median [range]			*p*-Value*
	Grade 1	Grade 2	Grade 3	
miR1246	3.76 (4.38); 3.38 [0,8.3]	1.46 (2.92); 0 [0,5.8]	7.04 (2.11); 7.78 [4,8.6]	0.0770
miR135a-5p	12.96 (0.79); 12.76 [12.3,14.1]	9.54 (6.43); 12.07 [0,14]	8.96 (1.62); 8.36 [7.8,11.4]	0.0627
miR137	6.06 (4.81); 7.13 [0,10]	6.24 (4.88); 6.59 [0,11.8]	0 (0); 0 [0,0]	0.0933
miR142-3p	12.14 (0.54); 12.28 [11.4,12.6]	10.84 (1.58); 10.98 [8.9,12.5]	13.23 (1.23); 13.46 [11.7,14.3]	0.0775
miR150-5p	10.62 (0.59); 10.8 [9.8,11.1]	9.5 (1.23); 9.74 [7.9,10.6]	11.74 (1.63); 11.78 [9.8,13.6]	0.0921
miR155-5p	6.59 (0.67); 6.38 [6.1,7.5]	5.04 (0.87); 4.95 [4.1,6.2]	8.33 (0.81); 8.36 [7.3,9.3]	**0.0173**
miR15b-5p	11.53 (0.62); 11.4 [10.9,12.4]	10.93 (0.35); 10.89 [10.6,11.4]	12.13 (0.66); 12.06 [11.4,13]	**0.0374**
miR200a-3p	14.04 (0.21); 14.01 [13.8,14.3]	13.02 (1.43); 13.04 [11.3,14.7]	11.99 (1.4); 11.73 [10.8,13.7]	0.0775
miR216a-5p	6.56 (3.95); 6.63 [2,11]	1.96 (3.92); 0 [0,7.8]	2.05 (4.11); 0 [0,8.2]	0.0933
miR223-3p	10.8 (0.58); 10.88 [10.1,11.3]	9.23 (1.52); 9.32 [7.4,10.9]	11.02 (0.51); 10.98 [10.4,11.7]	0.0837
miR3151-5p	9.65 (1.33); 9.67 [8,11.3]	7.19 (4.87); 9 [0,10.8]	2.79 (3.72); 1.66 [0,7.9]	0.0569
miR33a-5p	4.17 (2.97); 4.99 [0,6.7]	2.36 (2.82); 1.9 [0,5.6]	0 (0); 0 [0,0]	0.0915
miR345-5p	6.44 (1.22); 6.02 [5.5,8.2]	4.72 (2.25); 4.6 [2.6,7.1]	1.18 (2.35); 0 [0,4.7]	**0.0477**
miR451a	15.7 (1); 15.81 [14.6,16.6]	13.76 (1.39); 13.64 [12.2,15.6]	14.6 (0.71); 14.28 [14.2,15.7]	0.0775
miR488-3p	6.5 (4.69); 8.02 [0,10]	5.84 (4.4); 6.67 [0,10]	0 (0); 0 [0,0]	0.0933
miR548d-5p**	6.59 (1.3); 6.2 [5.5,8.5]	5.1 (2.11); 4.91 [3.2,7.4]	0 (0); 0 [0,0]	**0.0201**
miR9-5p**	4.25 (4.91); 4.04 [0,8.9]	4.37 (2.97); 5.6 [0,6.3]	10.81 (2.44); 9.89 [9.1,14.4]	**0.0232**

*miRNAs with differential expression with $p<0.1$ were selected as candidates for further evaluation of differential expression between grades. Specific miRNAs with $p<0.05$ have their p-value bolded. **miR9-5p and miR548d-5p were the most consistent in demonstrating statistically significant differential expression ($p<0.05$) among all comparisons across different grades of NENs.

Table II. *Comparison of miRNAs with statistically significant differential expression between grade 1 and 2 pancreatic neuroendocrine tumours (NETs).*

miRNA	Grade, mean (standard deviation); median [range]		*p*-Value*
	Grade 1	Grade 2	
miR1285-5p	6.4 (1.39); 6.09 [5.1,8.3]	3.36 (2.27); 4.29 [0,4.9]	**0.0304**
miR335-5p	9.29 (1.13); 9.33 [7.9,10.6]	7.58 (0.43); 7.66 [7,8]	0.0606

*miRNAs with differential expression with $p<0.1$ were selected as candidates for further evaluation of differential expression between grades. Specific miRNAs with $p<0.05$ have their *p*-value bolded.

(Figure 1 and Figure 2). Finally, 5 miRNAs (miR155-5p, miR15b-5p, miR222-3p, miR548d-5p, and miR9-5p) demonstrated a statistically significant differential expression ($p<0.05$) between G2 PNETs and G3 PNENs (Figure 3). MiR9-5p and miR548d-5p were the only 2 miRNAs that consistently maintained statistically significant differential expression ($p<0.05$) when compared across 3 separate groups (G1 and G3 PNENs, G2 and G3 PNENs, and G1, G2 and G3 PNENs). MiR9-5p and miR548d-5p also demonstrated *p*-values that were recurrently lower than the majority of the other miRNAs that they were compared with. MiR15b-5p and miR155-5p maintained statistically significant differential expression ($p<0.05$) when compared across G2 and G3 PNENs and G1, G2 and G3 PNENs. miR-345-5p maintained statistically significant differential expression ($p<0.05$) when compared across G1 and G3 PNENs and G1, G2 and G3 PNENs. 3 miRNAs (miR3151-5p, miR488-3p and miR33a-5p) had $p>0.05$ but were noteworthy because of their significant degree of consecutive down-regulation with increasing PNET grades.

The 24 miRNAs in Table I, Table II, Table III, and Table IV were further evaluated for survival association in pancreatic cancer using Oncomir (http://www.oncomir.org/oncomir/survival_custom.html). Two miRs showed a *p*-value <0.05 by log-rank test: miR-33a-5p ($p=0.037$) and miR-488-3p ($p=0.002$) based on median cut-off of miRNA expression. Specifically, patients with higher miR-33a-5p or miR-488-3p expression had better overall survival compared to those with lower expression, suggesting potential tumour suppressive roles with higher expression for improved survival (Figure 4 and Figure 5).

Table III. *Comparison of miRNAs with statistically significant differential expression between grade 1 and 3 pancreatic neuroendocrine neoplasms (NENs).*

miRNA	Grade, mean (standard deviation); median [range]		*p*-Value*
	Grade 1	Grade 3	
miR129-5p	8.95 (1.62); 8.91 [7,11]	5.24 (3.78); 6.08 [0,8.8]	0.0606
miR135a-5p	12.96 (0.79); 12.76 [12.3,14.1]	8.96 (1.62); 8.36 [7.8,11.4]	**0.0304**
miR137	6.06 (4.81); 7.13 [0,10]	0 (0); 0 [0,0]	0.0689
miR146b-5p	5.98 (0.67); 6 [5.3,6.6]	7.56 (0.71); 7.85 [6.5,8.1]	0.0814
miR155-5p	6.59 (0.67); 6.38 [6.1,7.5]	8.33 (0.81); 8.36 [7.3,9.3]	0.0606
miR200a-3p	14.04 (0.21); 14.01 [13.8,14.3]	11.99 (1.4); 11.73 [10.8,13.7]	**0.0304**
miR29c-3p	13.51 (0.35); 13.48 [13.1,14]	12.38 (0.94); 12.43 [11.4,13.3]	0.0606
miR3151-5p	9.65 (1.33); 9.67 [8,11.3]	2.79 (3.72); 1.66 [0,7.9]	**0.0294**
miR33a-5p	4.17 (2.97); 4.99 [0,6.7]	0 (0); 0 [0,0]	0.0689
miR345-5p	6.44 (1.22); 6.02 [5.5,8.2]	1.18 (2.35); 0 [0,4.7]	**0.0265**
miR488-3p	6.5 (4.69); 8.02 [0,10]	0 (0); 0 [0,0]	0.0689
miR548d-5p**	6.59 (1.3); 6.2 [5.5,8.5]	0 (0); 0 [0,0]	**0.0211**
miR628-5p	6.09 (1.13); 6.6 [4.4,6.8]	1.91 (2.26); 1.59 [0,4.5]	0.0591
miR9-5p**	4.25 (4.91); 4.04 [0,8.9]	10.81 (2.44); 9.89 [9.1,14.4]	**0.0294**

*miRNAs with differential expression with $p<0.1$ were selected as candidates for further evaluation of differential expression between grades. Specific miRNAs with $p<0.05$ have their *p*-value bolded. **miR9-5p and miR548d-5p were the most consistent in demonstrating statistically significant differential expression ($p<0.05$) among all comparisons across different grades of NENs.

Table IV. *Comparison of miRNAs with statistically significant differential expression between grade 2 and 3 pancreatic neuroendocrine neoplasms (NENs).*

miRNA	Grade, mean (standard deviation); median [range]		*p*-Value*
	Grade 2	Grade 3	
miR1246	1.46 (2.92); 0 [0,5.8]	7.04 (2.11); 7.78 [4,8.6]	0.0545
miR137	6.24 (4.88); 6.59 [0,11.8]	0 (0); 0 [0,0]	0.0689
miR142-3p	10.84 (1.58); 10.98 [8.9,12.5]	13.23 (1.23); 13.46 [11.7,14.3]	0.0606
miR155-5p	5.04 (0.87); 4.95 [4.1,6.2]	8.33 (0.81); 8.36 [7.3,9.3]	**0.0304**
miR15b-5p	10.93 (0.35); 10.89 [10.6,11.4]	12.13 (0.66); 12.06 [11.4,13]	**0.0304**
miR222-3p	9.93 (0.37); 10.11 [9.4,10.1]	11.37 (0.82); 11.35 [10.4,12.3]	**0.0304**
miR223-3p	9.23 (1.52); 9.32 [7.4,10.9]	11.02 (0.51); 10.98 [10.4,11.7]	0.0606
miR488-3p	5.84 (4.4); 6.67 [0,10]	0 (0); 0 [0,0]	0.0689
miR548d-5p**	5.1 (2.11); 4.91 [3.2,7.4]	0 (0); 0 [0,0]	**0.0211**
miR9-5p**	4.37 (2.97); 5.6 [0,6.3]	10.81 (2.44); 9.89 [9.1,14.4]	**0.0304**

*miRNAs with differential expression with $p<0.1$ were selected as candidates for further evaluation of differential expression between grades. Specific miRNAs with $p<0.05$ have their *p*-value bolded for emphasis. **miR9-5p and miR548d-5p were the most consistent in demonstrating statistically significant differential expression ($p<0.05$) among all comparisons across different grades of NENs.

Discussion

There has been an evolving appreciation of the importance and diversity of the regulatory roles that non-coding RNAs (ncRNAs) play in normal cellular function and the consequences of ncRNA dysregulation in cancer development (34, 35). MiRNAs represent a significant portion of ncRNAs and have been shown to play a role in oncogenesis and metastasis in the context of transcriptomic dysregulation (36-40).

Previous studies have demonstrated that some miRNAs have behaviour that is oncogenic when up-regulated (*i.e.*, oncomirs) or down-regulated (*i.e.*, tumour-suppressive anti-oncomirs). Some miRNAs can function as either tumour-suppressive or oncogenic depending on which tumour type they are found to be dysregulated in. MiRNAs are generally non-stringent regarding the targets that they bind to; consequently, it is not uncommon for an miRNA that predominantly functions as an oncomir in most contexts to occasionally serve as an anti-oncomir. Conversely, an anti-oncomir in most contexts could occasionally serve as an oncomir (41).

Previous studies have characterized DEMs within tumour types as a basis for miRNA profiles that may have potential

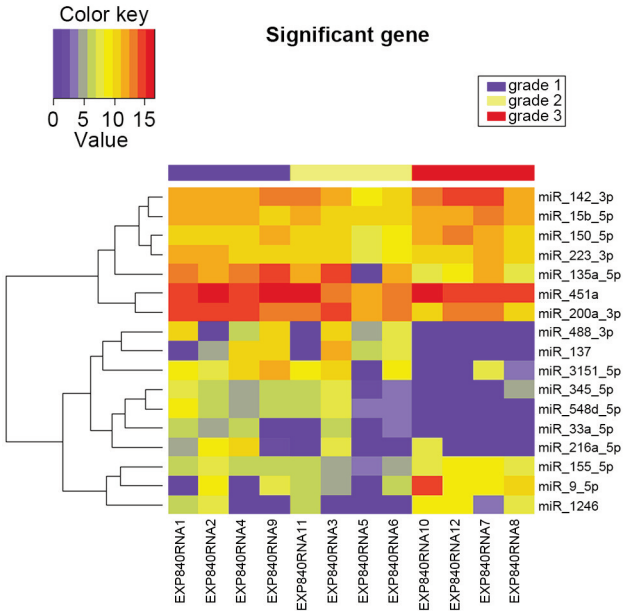

Figure 1. *Comparison of miRNAs with statistically significant differential expression between grade 1, 2 and 3 pancreatic neuroendocrine neoplasms (PNENs). miR9-5p and miR548d-5p were the most consistent in statistically significant differential expression (p<0.05) among comparisons across different grades of PNENs. Please note that miRNA data are normalized using log2-scale gene expression.*

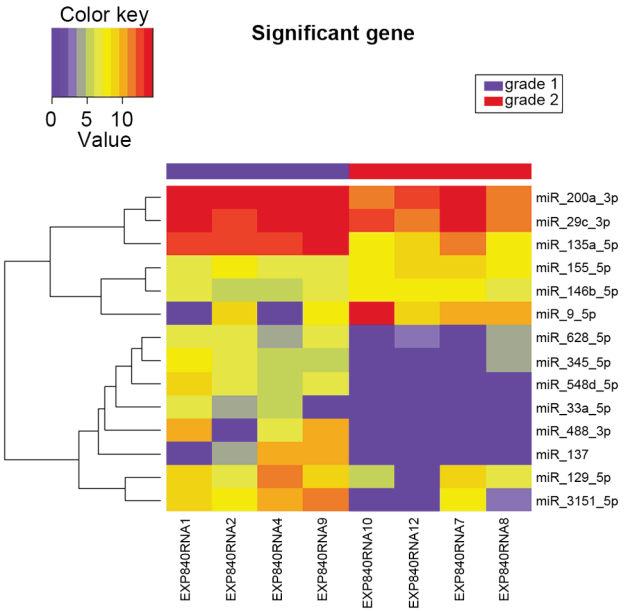

Figure 2. *Comparison of miRNAs with statistically significant differential expression between grade 1 and 3 pancreatic neuroendocrine neoplasms (PNENs). miR9-5p and miR548d-5p were the most consistent in statistically significant differential expression (p<0.05) among comparisons across different grades of PNENs. Please note that miRNA data are normalized using log2-scale gene expression.*

clinical utility (42). Likewise, the goal of our study was to aid in the development of an miRNA profile that has potential clinical utility for accurately grading PNENs.

Candidate miRNAs. Candidate miRNAs were identified from the miRNA expression profiles of 800 miRNAs. miR9-5p and miR548d-5p most consistently maintained significant differential expression across the 3 groups of comparisons. MiR-345-5p, miR15b-5p and miR155-5p were the next most consistently maintained, with statistically significant differential expression in 2 groups.

miR9-5p. MiR9-5p consistently functioned as an oncomir in our study, with consecutively up-regulated expression with each increase in PNEN grade. MiR-9-5p up-regulation in breast cancer has been shown to affect FOXO1, LIFR, and E-Cadherin expression, inducing resistance to therapy with alkylating agents (43, 44) and tamoxifen (43-45) and consequently increasing metastatic potential and negatively affecting patient outcomes. miR-9-5p up-regulation also correlates with increased metastatic potential and tumour recurrence of head and neck cancer (45, 46).

miR548d-5p. MiR548d-5p functioned as an anti-oncomir in our study, with consecutively down-regulated expression

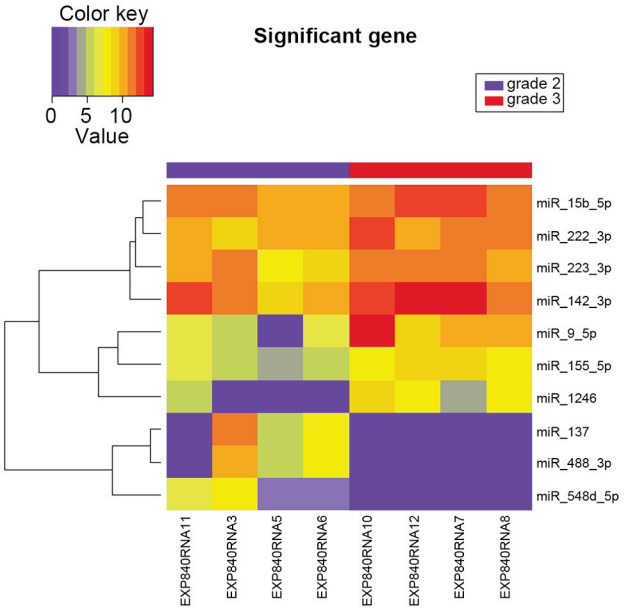

Figure 3. *Comparison of miRNAs with statistically significant differential expression between grade 2 and 3 pancreatic neuroendocrine neoplasms (PNENs). miR9-5p and miR548d-5p were the most consistent in statistically significant differential expression (p<0.05) among comparisons across different grades of PNENs. Please note that miRNA data are normalized using log2-scale gene expression.*

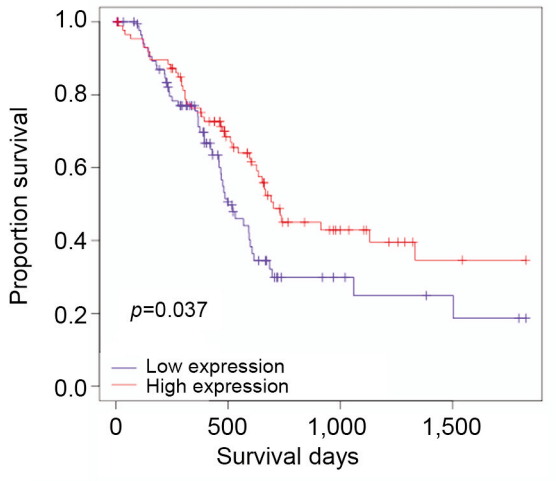

Figure 4. *Kaplan–Meier plot of overall survival for miR-33a-5p in pancreatic adenocarcinoma patients and the cohort with a score below median, labelled as low-expression. The figure was generated using the KM Plotter Online Tool.*

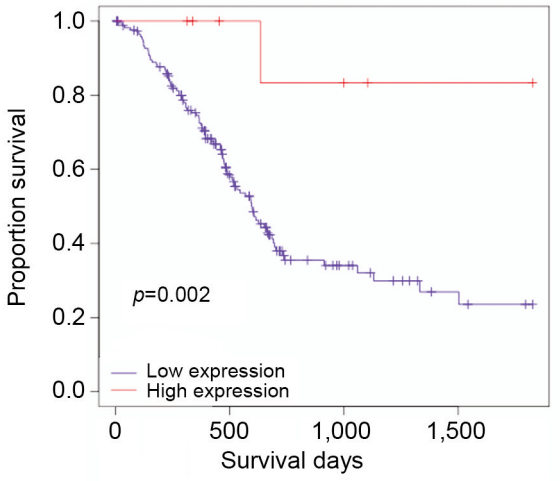

Figure 5. *Kaplan–Meier plot of overall survival for miR-488-3p in pancreatic adenocarcinoma patients and the cohort with a score below median was labelled as low-expression. The figure was generated using the KM Plotter Online Tool.*

with each increase in PNEN grade. The results of previous studies corroborate that most members of the miR548 family typically behave in a tumour-suppressive manner (47-49). MiR548d-5p also has been proposed as a potential biomarker for responsiveness to neoadjuvant chemoradiotherapy (50). Preclinical data putatively support miR548d-5p having an inhibitory effect on *PTPN12* (51). MiR548d-5p behaves as an anti-oncomir in pancreatic cancer, inhibiting growth and enhancing sensitization to gemcitabine (52). Interestingly, an miRNA that is homologous to miR548d-5p has been reported to be located within the fourth intron within the tumour suppressor gene *FHIT* (53).

miR-345-5p. MiR-345-5p likewise functioned as an anti-oncomir in our study, with consecutively down-regulated expression with each increase in PNEN grade. MiR-345-5p down-regulation in non–small cell lung cancer was associated with progression and poor prognosis (54). It has been shown that up-regulation of miR-345-5p in gastric cancer affects the migration and metastatic potential of gastric cancer cells by modulating *FOXQ1* expression and epithelial-the mesenchymal transition (55). MiR-345-5p down-regulation in pancreatic cancer has shown to reduce apoptosis both through activation of caspase-dependent and caspase-independent pathways and by modulating *BCL2* (56). In another study MiR345,5p was down-regulated and caused suppression of pancreatic cancer proliferation and metastasis by targeting CCL8 and NF-kB (57).

MiR-345-5p inhibits proliferation and metastatic potential of colon cancer cells by targeting *BAG3* and is shown to be down-regulated in colorectal cancer (58). It is also down-regulated in hepatocellular carcinoma, where down-regulation correlates with a significantly decreased overall survival rate, and targets *YAP1* (59). MiR-345-5p suppresses proliferation, migration, and invasion of prostate cancer cells by targeting *SMAD1*. Alternatively, miR-345-5p promotes oncogenic growth and migration of castration-resistant prostate cancer cells by inhibiting the tumour-suppressive activity of *CDKN1A* (60, 61).

miR15b-5p. MiR15b-5p functioned as an oncomir in our study, with up-regulated expression with the overall comparison of G1 PNETs to G3 PNENs, though down-regulation was observed when comparing G2 with G1. These findings are concordant with studies showing that up-regulation of miR15b-5p in gastric cancer promotes proliferation, migration, invasion, epithelial-mesenchymal transition, and metastasis by targeting the tumour suppressor gene *PAQR3* (62, 63). Up-regulation of miR-15b in colorectal cancer down-regulates *MTSS1* and Klotho proteins, correlating with metastasis, recurrence, and poor patient prognosis (64). Low miR-15b expression suppresses cell growth and induces apoptosis of hepatocellular carcinoma cells; improved overall survival rates were shown in patients with low miR-15b expression. Moreover, up-regulated miR-15b is a predictor of a worse prognoses for patients with hepatocellular carcinoma after curative hepatectomy (65). MiR-15b-5p was also found to be up-regulated in prostate cancer and exerts a tumorigenic effect by targeting the tumour suppressor gene *RECK* and facilitating tumour recurrence (66). MiR-15b-5p is also up-regulated in the transformation from classical to aggressive Mantle cell lymphoma (67) and in squamous cell carcinoma of the head and neck (68).

miR155-5p. MiR155-5p functioned as an oncomir in our study, with up-regulated expression with the overall comparison of G1 PNETs to G3 PNENs. Similar to miR15b-5p, miR155-5p was down-regulated when comparing G2 to G1.

MiR-155-5p has been described as a hypoxia-inducible oncomir that is mediated by HIF1α, wherein miR-155-5p down-regulates *ELK3* (69). In colorectal cancer, miR-155-5p up-regulation is correlated with poor prognoses, and miR-155-5p posttranscriptional regulation of human antigen R promotes migration of colorectal cancer cells (70). Cancer cells also produce miR155 to convert normal fibroblasts to cancer associated fibroblasts (71) contributing to the EMT associated phenotype (72). Increased expression of miR-155 by pancreatic cancer was found to be an early event in the transition from non-neoplastic pancreatic tissue to intraductal pancreatic mucinous neoplasm (IPMN), a precursor of pancreatic adenocarcinoma. In their study, Hobbe *et al*. found such an increase in miR-155 to be present not only in pancreatic IPMN tissue but also in the fluid derived by pancreatic cysts harbouring IPMN. The authors suggested that miR-155 may be used as a biomarker to identify IPMN (73). MiR155-5p up-regulation in breast cancer is significantly decreased following surgical resection (74). A recent preclinical study demonstrated that inhibition of miR-155-5p subsequently prevented the formation of breast cancer stem cells (75). MiR-155-5p up-regulation in renal carcinoma promotes proliferation and invasion *via* inhibition of glycogen synthase kinase-3β (GSK-3β), which in turn up-regulates the Wnt/β-catenin signalling pathway (76). In diffuse large B-cell lymphoma, up-regulation inhibits *SOCS3*, which suppresses apoptosis and up-regulates the JAK-STAT3 signalling pathway (77). In a murine model, inhibiting miR-155-5p delayed diffuse large B-cell lymphoma progression (78).

MiR-155-5p can either up-regulate or down-regulate the mTOR pathway depending on the tumour type. In osteosarcoma, miR-155-5p inhibits *PTEN*, consequently up-regulating the mTOR pathway (79). Conversely, a recent study reported tumour-suppressive behaviour of miR-155-5p *via* inhibition of PDK1 and subsequent down-regulation the mTOR pathway in cervical cancer (80). MiR-155-5p down-regulation has also been shown to be tumour-suppressive by sensitizing gastric cancer cells to cisplatin (81). MiR-155-5p may have implications for immunotherapy, as it suppresses expression of programmed death ligand-1 (PD-L1) (82).

miR3151-5p, miR488-3p, and miR33a-5p. MiR3151-5p, miR488-3p, and miR33a-5p had p-values >0.05 and demonstrated consecutive down-regulation with increasing PNEN grade. A review of corroborating studies support potential tumour suppressive roles of all 3 of these miRNAs, including miR3151-5p down-regulation in chronic lymphocytic leukaemia; miR488-3p down-regulation in NETs of the small intestine and in gliomas; and miR33a-5p down-regulation in lung adenocarcinoma, osteosarcoma, melanoma, and prostate cancer (25, 83, 84).

It is also interesting that when all of the 24 miRNAs in Table I, Table II, Table III, and Table IV were evaluated for survival association in pancreatic adenocarcinoma using Oncomir (http://www.oncomir.org/oncomir/survival_custom.html), two miRs showed a p-value <0.05 by log-rank test: miR-33a-5p (p=0.037) and miR-488-3p (p=0.002), based on median cut-off of miRNA expression. Specifically, patients harbouring pancreatic ductal adenocarcinoma with higher miR-33a-5p or miR-488-3p expression had better overall survival compared to those with lower expression, suggesting potential tumour suppressive roles of these miRNA across different pancreatic tumour types.

Conclusion

The observed differential expression of the miRNAs in our study are concordant with patterns of dysregulation that have been established in prior studies of different tumour types. The aforementioned miRNA targets that have been reported in the literature may serve as a reference for investigating potential targets that may be responsible for the dysregulation observed in different grades of PNENs.

The reliability of these candidate miRNAs with statistically significant differential expression between PNEN grades supports further investigation in a larger population of patients, with the goal of identifying potential prognostic utility by providing clarity for PNENs with significant grading challenges. Furthermore, if future studies further corroborate the reliability of these candidate miRNAs for PNEN grading, a profile comprised of these miRNAs may have potential as an independent prognostic biomarker to accurately grade PNENs.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

Dr. James Saller collected the samples, He also collated the data and drafted the manuscript. Daley White contributed to the interpretation of data as well as drafting and critical revision of the manuscript; Brooke Hough helped with the literature search and review. Dr. Sean Yoder performed the NanoString analysis and evaluated the results, Drs. Junmin Whiting and Dung-Tsa Chen performed the statistical analysis. Dr. Anthony Magliocco supervised the analysis and critically reviewed the data. He also reviewed the final form of the manuscript. Dr. Domenico Coppola designed the study, critically evaluated the data, and reviewed the final form of the manuscript.

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