Prognostic relevance of proliferation-related miRNAs in pancreatic neuroendocrine neoplasms

Vince Kornél Grolmusz^{1,2,3}, Annamária Kövesdi^{1,2}, Katalin Borka⁴, Peter Igaz^{1,5} and Attila Patócs^{2,5,6}

¹2nd Department of Medicine, Semmelweis University, Budapest, Hungary, ²'Lendület' Hereditary Endocrine Tumours Research Group, Hungarian Academy of Sciences – Semmelweis University, Budapest, Hungary,

³Department of Medical Oncology and Therapeutics, Beckman Research Institute, City of Hope National Medical Center, Monrovia, California, USA, ⁴2nd Department of Pathology, Semmelweis University, Budapest, Hungary,

⁵MTA-SE Molecular Medicine Research Group, Hungarian Academy of Sciences – Semmelweis University, Budapest, Hungary, and ⁶Department of Laboratory Medicine, Semmelweis University, Budapest, Hungary

Correspondence
should be addressed
to A Patócs

Email
patocs.attila@med.
semmelweis-univ.hu

Abstract

Objective: Pancreatic neuroendocrine neoplasms (PanNENs) are rare tumors arising from the endocrine pancreas; however, their prognosis differs significantly upon their proliferative state, which is characterized by histopathological grading. MiRNAs are small, noncoding RNAs posttranscriptionally regulating gene expression. Our aim was to identify miRNAs with altered expression upon proliferation which can be used as prognostic biomarkers in PanNENs.

Methods: MiRNA expression profiles of 40 PanNENs were downloaded from Gene Expression Omnibus and were reanalyzed upon tumor grades (discovery cohort). Results of the reanalysis were confirmed by qRT-PCR analysis of five miRNAs on an independent validation cohort of 63 primary PanNEN samples. Cox proportional hazards survival regression models were fit for both univariate and multivariate analysis to determine the miRNAs' effect on progression-free and overall survival.

Results: Nineteen miRNAs displayed differential expression between tumor grades. The altered expression of three out of five chosen miRNAs was successfully validated; hsa-miR-21, hsa-miR-10a and hsa-miR-106b were upregulated in more proliferative PanNENs compared to Grade 1 tumors. In univariate analysis, higher expression of tissue hsa-miR-21, hsa-miR-10a and hsa-miR-106b of primary PanNENs predicted worse progression-free and overall survival; however, multivariate analysis only confirmed the expression of hsa-miR-21 as an independent prognostic factor.

Conclusions: The expression of hsa-miR-106b, hsa-miR-10a and especially hsa-miR-21 has prognostic relevance regarding progression-free and overall survival in patients with PanNENs.

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Introduction

Pancreatic neuroendocrine neoplasms (PanNENs) are rare tumors arising from the endocrine islets of the pancreas with an incidence of 0.2–0.3/100 000 (1, 2). In the majority of the cases, PanNENs occur sporadically; however, some hereditary tumor syndromes (e.g. multiple endocrine neoplasia type 1, von Hippel-Lindau syndrome, neurofibromatosis type 1, tuberous sclerosis) might also result in PanNEN formation (3). Histological classification

of these tumors is based on their proliferative nature assessed by the Ki-67 index and mitotic count (4). Upon the WHO classification, Grade 1 and Grade 2 tumors are less proliferative, while the prognosis of Grade 3 tumors is very poor (4).

High throughput analyses contributed to enlighten the molecular background of PanNENs (5, 6). A whole exome sequencing analysis demonstrated frequent

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mutations in genes implicated in chromatin remodeling (*MEN1*, *DAXX*, *ATRX*), and mutations in other well-known tumor suppressor genes (*PTEN*, *TSC2*) were also detected (5). Additionally, a gene expression microarray study performed on a large sample set confirmed the decreased expression of mTOR pathway-inhibiting *PTEN* and *TSC2* contributing to PanNEN formation (6). Most recently, a large scale whole-genome sequencing study found that germline mutations of DNA repair genes *MUTYH*, *CHEK2* and *BRCA2* might also be found in clinically sporadic cases (7).

miRNAs are small, 21–26 nt long single-stranded RNA molecules regulating gene expression at the post-transcriptional level via RNA interference (8). Several layers of evidence confirmed the role of tissue-specific miRNA expression alterations in the pathogenesis of various tumors (9). As miRNAs are chemically more stable compared to mRNAs, miRNA expression can optimally be measured from formalin-fixed, paraffin-embedded samples (10, 11, 12), as it correlates well with expression levels obtained from fresh frozen specimens (11).

To date, only two studies examined the PanNEN miRNA expression profiles (13, 14). These single cohort studies proposed the well-known onco-miR hsa-miR-21 to have elevated expression in highly proliferative, metastatic PanNENs (13), while the expression of hsa-miR-642 was found to be correlated with Ki-67 score and hsa-miR-210 correlated with metastatic disease (14).

Although the reanalysis of previous miRNA microarray experiments revealed three clusters of PanNENs upon miRNA expression signature (15), the prognostic significance of these clusters or single miRNAs has not been thoroughly investigated. Only one study proposed prognostic relevance of hsa-miR-196a to be associated with poorer prognosis in a sample of 37 PanNENs (16).

Here, we present a reanalysis of previous miRNA profiling data and validate our findings on an independent, larger dataset containing 63 primary tumors revealing miRNAs of prognostic importance in PanNENs.

Subjects and methods

In silico reanalysis of publicly available miRNA microarray data

Previously published miRNA expression profiles were downloaded from Gene Expression Omnibus (GEO ID: GSE73350) (13) and were reanalyzed using GeneSpring 12.6 (Agilent Technologies) software. In total, 40 miRNA

expression arrays of PanNEN tissues were included (discovery cohort).

PanNEN patients and tissues

Sixty three independent, primary PanNEN samples were enrolled between 1999 and 2015 at the 2nd Department of Pathology, Semmelweis University (validation dataset). Consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used. All patients were of Caucasian origin. Routine pathological examination followed by histopathological analyses, including Ki-67 immunohistochemistry analysis and determination of the mitotic count performed by a pathologist expert in endocrine pathology (K B) confirmed the diagnosis of PanNEN. Ki-67 immunohistochemistry was performed as earlier reported using the monoclonal mouse anti-Ki-67 antibody (Clone MIB-1, Dako Cat. No.: M7240) (10). Grading of PanNEN samples was performed according to the WHO 2017 classification with the grade denoting the higher value of Ki-67 index and mitotic count (17). The study was approved by the Scientific and Research Committee of the Medical Research Council of Hungary (ETT-TUKEB 4457/2012/EKU).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from formalin-fixed, paraffin-embedded sections using Ambion RecoverAll Total Nucleic Acid Isolation kit for FFPE tissues (Thermo Fisher Scientific) as earlier reported (10). Five nanograms of total RNA was reverse transcribed using single tube TaqMan microRNA assays (assay IDs: hsa-miR-21: 000397; hsa-miR-29a: 002112; hsa-miR-10a: 000387; hsa-miR-106b: 000442; hsa-miR-101: 002253; RNU6B: 001093) and TaqMan microRNA reverse transcription kit (Thermo Fisher Scientific) while miRNA expression was quantified on a 7500 Fast Real-time PCR instrument (Thermo Fisher Scientific) as earlier reported (10).

MiRNA expression was normalized to the relative expression of RNU6B (ΔCt method) (10). All measurements were performed in triplicate.

Statistical analysis

Statistical analysis of the microarray data was performed using GeneSpring 12.6 (Agilent Technologies) software as described earlier (18). Following the manufacturer's recommendation, total signal normalization at the 75th

percentile of raw signal values and baseline transformation at the median of all samples were performed as described earlier (18). Differentially expressed miRNAs upon tumor grade (Grade 1, Grade 2, Grade 3) were detected by one-way ANOVA followed by Tukey's Honestly Significant Difference *post hoc* test and Benjamini–Hochberg correction for multiple measurements (18). Hierarchical clustering was performed on the Morpheus online tool (URL: <https://software.broadinstitute.org/morpheus/>) using the one minus Pearson correlation method.

The results of qRT-PCR experiments were analyzed using GraphPad Prism version 6 software. The normality of distribution was confirmed by Shapiro–Wilk normality test. For examining miRNA expression differences between tumor grades ($-\Delta\text{Ct}$ values), one-way ANOVA test and subsequent *post hoc* Tukey's multiple comparisons test was used. For examining miRNA expression differences between patients with non-metastatic and metastatic disease ($-\Delta\text{Ct}$ values), Student's independent samples *T*-test was used. For significantly differently expressed miRNAs both in the discovery and validation cohorts, Cox proportional hazards survival regression models were fit for both univariate and multivariate analysis to determine their effects on progression-free and overall survival and also on the time to disease progression.

In all comparisons, *P* value <0.05 was considered statistically significant.

Results

In silico reanalysis and subsequent validation identifies several miRNAs to be differently expressed upon tumor grade in PanNENs

Upon the *in silico* reanalysis in the discovery cohort, 19 miRNAs, including the well-known onco-miR hsa-miR-21, were found to have altered expression between tumor grade in PanNENs (Fig. 1, Panel A). Hierarchical clustering upon the expression profiles of differentially expressed miRNAs rendered the samples in two clusters differing in proliferative nature: C1 cluster-incorporated eighteen Grade 1 and four Grade 2 tumors, while C2 consisted of five Grade 1, eleven Grade 2 and two Grade 3 tumors (Fig. 1, Panel A).

Five miRNAs (hsa-miR-21, hsa-miR-29a, hsa-miR-10a, hsa-miR-106b and hsa-miR-101) were chosen for validation in the independent validation cohort containing 28 Grade 1, 27 Grade 2 and 8 Grade 3 tumors (Table 1). The validation cohort contained 44 non-functioning tumors,

17 insulinomas, one gastrinoma and one somatostatinoma and 38 out of 63 of the patients were female. The mean age of the patients was 54.4 ± 17.3 years. Among the five chosen miRNAs, hsa-miR-21, hsa-miR-10a and hsa-miR-106b were differently expressed between tumor grades and all three were upregulated in more proliferative PanNENs (Fig. 1, Panel B).

Figure 1

Discovery and validation of novel miRNAs differently expressed upon tumor grade in PanNENs. Panel (A): Heat map and hierarchical clustering of significantly differently expressed miRNAs upon tumor grade in the *in silico* reanalysis of the previously published dataset by Roldo *et al.* (13). Panel (B): Validation of the expression of five chosen miRNAs (hsa-miR-21, hsa-miR-29a, hsa-miR-10a, hsa-miR-106 and hsa-miR-101) in the validation cohort by qRT-PCR measurements. Higher $-\Delta\text{Ct}$ levels correspond to higher miRNA expression. Bars indicate mean $\pm$ standard deviation (s.d.). Statistics: one-way ANOVA followed by Tukey's multiple comparisons test. Asterisks mark statistical significance ($P < 0.05$). A full colour version of this figure is available at <https://doi.org/10.1530/EJE-18-0305>.

Table 1 Baseline characteristics of PanNENs involved in the validation cohort.

ID	Sex	Age (years)	Familial or sporadic	Symptoms at diagnosis	Clinical diagnosis	Largest diameter (mm)	Ki67 index (%)	Mitotic cells (/10HPF)	WHO grade	Metastasis at the time of diagnosis	Duration of follow-up (days)	Disease status at the time of study
1	M	51	Sporadic	Weight loss	Non-functioning tumor	40	1	1	1		3988	Disease free
2	F	60	Sporadic		Non-functioning tumor	35	1	1	1	Local lymph node and liver	5339	Disease progression
3	F	45	Sporadic		Non-functioning tumor	70	1	0	1	n.a.		
4	M	66	Sporadic	Incidental	Non-functioning tumor	40	1	1	1		1925	Death
5	F	73	Sporadic	Abdominal pain, weight loss	Non-functioning tumor	14	2	1	1		1214	Death
6	M	75	Sporadic	Recurring hypoglycemia	Insulinoma	22	<0.5	0	1		4585	Death
7	F	76	Sporadic		Non-functioning tumor	34	1	1	1		8	Death
8	M	58	Sporadic		Insulinoma	10	1	0	1	n.a.		
9	F	57	Sporadic	Recurring hypoglycemia	Insulinoma	10	1	0	1		4880	Disease free
10	F	37	Sporadic	Incidental	Non-functioning tumor	60	1	1	1		4158	Disease free
11	F	81	Sporadic	Hypoglycemia	Insulinoma	12	2	1	1		736	Death
12	M	56	Sporadic	Incidental	Non-functioning tumor	35	1	0	1			
13	M	70	Sporadic		Insulinoma	30	1	1	1		4150	Death
14	M	49	Sporadic		Insulinoma	16	1	0	1			
15	F	47	Sporadic	Recurring hypoglycemia	Insulinoma	30	1	0	1		4826	Disease free
16	M	65	Sporadic	Recurring hypoglycemia	Insulinoma	18	2	0	1			
17	F	81	Sporadic	Recurring hypoglycemia	Insulinoma	15	1	1	1		1800	Disease free
18	M	87	Sporadic	Recurring hypoglycemia	Insulinoma	15	1.5		1		871	Death
19	F	24	Sporadic	Recurring hypoglycemia	Insulinoma	15	1	0	1			
20	F	71	Sporadic	Recurring hypoglycemia	Non-functioning tumor	38	2	0	1			
21	F	46	Sporadic	Abdominal pain	Non-functioning tumor	30	1	1	1	Local lymph node and liver	3252	Disease progression
22	F	74	Sporadic	Incidental	Non-functioning tumor	10	0.5	0	1		1572	Disease free

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23	M	56	Sporadic	Incidental	Non-functioning tumor	45	<0.5	1	1368	Disease free	
24	F	50	Sporadic	Abdominal discomfort	Non-functioning tumor		1.5	1	1273	Disease free	
25	F	26	MEN1-associated	Abdominal pain	Non-functioning tumor	110	<0.5	1	6677	Tumor recurrence	
26	F	24	MEN1-associated	Recurring hypoglycemia	Insulinoma	30	1	1	1586	Disease free	
27	F	21	MEN1-associated	Hypoglycemia	Insulinoma	15	1.5	1	2786	Tumor recurrence	
28	M	46	Sporadic	Recurring hypoglycemia	Insulinoma	15	1	1			
29	M	77	Sporadic	Weight loss	Non-functioning tumor	35	15	1	1291	Death	
30	F	49	Sporadic		Non-functioning tumor	135	3	6	4689	Disease progression	
31	M	55	Sporadic	Incidental	Non-functioning tumor	26	1	2	4571	Disease free	
32	F	53	Sporadic		Non-functioning tumor	50	5	4	2530	Death	
33	F	36	Sporadic	Abdominal discomfort	Somatostatinoma	50	5	1			
34	M	38	Sporadic		Non-functioning tumor	130	15	2	4451	Disease progression	
35	M	55	Sporadic	Incidental	Gastrinoma	40	7.5	1			
36	F	73	Sporadic	Abdominal discomfort	Non-functioning tumor	28	10	3	3797	Disease progression	
37	F	39	Sporadic	Abdominal discomfort	Non-functioning tumor	70	10	5			
38	F	70	Sporadic	Abdominal discomfort	Non-functioning tumor	80	3	2	3179	Death	
39	F	62	Sporadic	Abdominal pain	Non-functioning tumor	40	4	2	2897	Tumor recurrence	
40	F	55	Sporadic	Recurring hypoglycemia	Insulinoma	25	3	0	4624	Disease free	
41	F	70	Sporadic		Non-functioning tumor	35	3	1	4510	Disease free	
42	M	51	Sporadic	Diarrhea	Non-functioning tumor	95	15	3	2973	Disease progression	
43	F	53	Sporadic	Weight loss, diarrhea	Non-functioning tumor	52	3	1	2644	Disease progression	
44	M	79	Sporadic	Incidental	Non-functioning tumor	18	5	0	1415	Death	
45	F	19	MEN1-associated	Recurring hypoglycemia	Insulinoma	18	4.5	2	3874	Disease free	
46	F	74	Sporadic	Recurring hypoglycemia	Insulinoma	16	3	0	1051	Death	
47	CF	40	Sporadic	Weight loss, diarrhea	Non-functioning tumor	28	1	3	5695	Disease progression	

(Continued)

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Table 1 Continued.

ID	Sex	Age (years)	Familial or sporadic	Symptoms at diagnosis	Clinical diagnosis	Largest diameter (mm)	Ki67 index (%)	Mitotic cells (/10HPF)	WHO grade	Metastasis at the time of diagnosis	Duration of follow-up (days)	Disease status at the time of study
48	M	66	Sporadic	Incidental	Non-functioning tumor	60	9	1	2		1446	Disease free
49	F	56	Sporadic	Incidental	Non-functioning tumor	30	4	0	2		2314	Stable disease
50	F	59	sporadic	Abdominal discomfort	Non-functioning tumor	20	3	1	2		2058	Disease free
51	F	52	Sporadic	Incidental	Non-functioning tumor	40	10	1	2			
52	F	38	Sporadic	Abdominal pain	Non-functioning tumor	56	2	2	2		3268	Disease progression
53	M	60	Sporadic	Incidental	Non-functioning tumor	37	7.5	2	2	Local lymph node	1260	Death
54	M	70	Sporadic	Abdominal pain	Non-functioning tumor	35	<0.5	5	2	Local lymph node		
55	M	63	Sporadic	Incidental	Non-functioning tumor		4	3	2	Local lymph node and liver		
56	F	55	Sporadic		Non-functioning tumor	10	80	52	3	Local lymph node	250	Death
57	M	74	Sporadic	Abdominal pain, weight loss	Non-functioning tumor	55	25	0	3	Local lymph node	1238	Death
58	M	68	Sporadic	Abdominal pain, weight loss	Non-functioning tumor	25	35		3	Local lymph node	455	Death
59	M	69	Sporadic	Jaundice	Non-functioning tumor		70	23	3	Local lymph node	329	Death
60	F	71	Sporadic	Weight loss, emesis	Non-functioning tumor	70	25	12	3	Local lymph node	569	Death
61	F	71	Sporadic	Jaundice	Non-functioning tumor	70	100	25	3	Local lymph node	196	Death
62	M	68	Sporadic	Weight loss	Non-functioning tumor		30		3	n.a.	616	Death
63	F	59	Sporadic	Weight loss, anorexia	Non-functioning tumor	25	70	50	3	Local lymph node and liver	352	Death

F, female; HPF, high power field; M, male; n.a., not applicable.

Expression analysis of hsa-miR-21, hsa-miR-10a and hsa-miR-106b between non-metastatic and metastatic disease

In our validation cohort, four patients had local lymph node and also liver, 14 patients had only local lymph node metastasis (totally 18 patients had metastatic disease) while 41 patients did not have metastasis at the time of diagnosis. We compared the expression levels of hsa-miR-21, hsa-miR-10a and hsa-miR-106b between patients with non-metastatic vs metastatic disease. Patients with metastatic disease had higher expression of hsa-miR-21 in the primary tumor ($-\Delta\text{Ct}(\text{non-metastatic}): 3.22 \pm 1.27$ vs $-\Delta\text{Ct}(\text{metastatic}): 4.08 \pm 1.64$ $P=0.0346$, note that the higher $-\Delta\text{Ct}$ values correspond to higher expression), while there were no differences in the expression levels of hsa-miR-10a ($-\Delta\text{Ct}(\text{non-metastatic}): -0.35 \pm 1.57$ vs $-\Delta\text{Ct}(\text{metastatic}): 0.47 \pm 2.30$ $P=0.1229$) and hsa-miR-106b ($-\Delta\text{Ct}(\text{non-metastatic}): 1.21 \pm 1.31$ vs $-\Delta\text{Ct}(\text{metastatic}): 1.80 \pm 1.70$ $P=0.1630$) in this regard.

Expression levels of hsa-miR-21, hsa-miR-10a and hsa-miR-106b have prognostic relevance in patients with PanNEN

Follow-up of 49 patients has been conducted for 7.01 ± 4.7 years. Univariate analysis revealed that hsa-miR-21, hsa-miR-10a and hsa-miR-106b are negative predictors of progression-free and overall survival; however, multivariate analysis confirmed only

hsa-miR-21 as an independent prognostic factor in PanNEN (Table 2, Panels A and B). Out of 49 patients during follow-up, 33 patients had disease progression (disease progression, tumor recurrence or death). In patients with disease progression, the expression levels of hsa-miR-10a and hsa-miR-106b were found to be negative predictors on the time to disease progression; however, no miRNAs were confirmed as independent prognostic factor (Table 2, Panel C).

Discussion

Due to their chemical stability, tissue specificity and pathogenic relevance, miRNAs are widely used biomarkers in health and disease. Minimally invasive sampling techniques facilitate early diagnosis and disease recurrence, which is of utmost importance regarding various diseases including cancer (19, 20). In particular, liquid biopsy from blood, saliva and urine may differentiate patients with different prognosis and contributes to histopathological diagnosis, as well (19, 20).

Although miRNA biomarkers are intensively sought in various types of cancer, only a few studies were directed to enlighten miRNA expression differences in PanNENs (13, 14, 16), and prognostic relevance of only hsa-miR-196a has been clearly proposed (16). Therefore, we aimed to reanalyze the results of the previously published and publicly available dataset of Roldo *et al.* (13) and validate our findings on the largest cohort in miRNA expression

Table 2 Survival analyses in the validation cohort. Follow-up information was available from 49 patients in the validation cohort. Cox proportional hazards survival regression models were fit for both univariate and multivariate analysis to determine the miRNA's effects on progression-free (Panel A, $n=49$) and overall survival (Panel B, $n=49$). Moreover, on patients with disease progression during follow-up additional Cox proportional hazards survival regression models were fit for both univariate and multivariate analysis to determine the miRNA's effects on time to disease progression (Panel C, $n=33$).

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-Value	HR (95% CI)	P-Value
A				
hsa-miR-21	1.04 (1.02–1.06)	0.0002	1.03 (1.01–1.05)	0.0120
hsa-miR-10a	1.10 (1.04–1.16)	0.0006	1.11 (0.89–1.38)	0.3556
hsa-miR-106b	1.04 (1.02–1.07)	0.0022	0.97 (0.87–1.07)	0.5249
B				
hsa-miR-21	1.04 (1.02–1.05)	0.0001	1.03 (1.00–1.05)	0.0313
hsa-miR-10a	1.16 (1.05–1.28)	0.0023	1.03 (0.80–1.34)	0.7997
hsa-miR-106b	1.07 (1.03–1.12)	0.0012	1.03 (0.91–1.16)	0.6428
C				
hsa-miR-21	1.01 (1.00–1.03)	0.1665	1.00 (0.98–1.02)	0.8792
hsa-miR-10a	1.08 (1.02–1.14)	0.0049	1.10 (0.82–1.46)	0.5246
hsa-miR-106b	1.04 (1.01–1.07)	0.0070	0.99 (0.86–1.14)	0.8981

Bold letters indicate statistically significant associations ($P < 0.05$).
95% CI, 95% confidence interval; HR, hazard ratio.

studies of PanNENs to date, specifically addressing the important issue of prognostic relevance.

Our *in silico* reanalysis revealed 19 miRNAs with altered expression between different tumor grades. Among these, the differential expression of hsa-miR-20a and hsa-miR-21 in PanNENs has been confirmed by an independent study, as well (14). The hierarchical clustering rendered the discovery cohort samples into two clusters upon the expression profile of these 19 miRNAs successfully creating the lesser proliferative C1 and the more proliferative C2 clusters. Upon the qRT-PCR results, three (hsa-miR-21, hsa-miR-10a and hsa-miR-106b) out of five chosen miRNAs has been successfully validated as overexpressed in more proliferative tumors in an independent larger cohort of PanNENs, confirming the biological relevance of our *in silico* analysis.

In the validation cohort, hsa-miR-21 had the largest fold increase between tumor grades with elevated expression in Grade 3 PanNENs. Since the previous two large sample miRNA expression studies also associated hsa-miR-21 expression with PanNEN formation and more aggressive biological behavior (13, 14), currently, this miRNA seems to be most tightly related to PanNEN tumorigenesis. Most recently, a large case-control study assessing tumor and plasma miRNA expression of 320 cases found in accordance, that hsa-miR-21 expression was higher in patients with metastatic small intestine neuroendocrine tumor than in healthy controls (21). Additionally, high hsa-miR-21 expression associated with shorter overall survival (21). Confirming previous studies (13, 21), we also showed that higher hsa-miR-21 expression in the primary tumor correlated with metastatic disease.

The importance of hsa-miR-21 as an onco-miR has been widely confirmed in several types of cancer (22); moreover, its targets have also been characterized (22), among which the negative correlation of hsa-miR-21 and its target programmed cell death 4 gene (*PDCD4*) has already been proposed by Roldo *et al.* (13). Moreover, the tumor suppressor protein *PDCD4* has been confirmed as a target of hsa-miR-21 in breast cancer cells (23) and other important pro-apoptotic factors (FasL and *PTEN*) have also been confirmed as direct targets of hsa-miR-21 (24). Therefore, the inhibition of this pro-apoptotic pathway might contribute to cell survival and proliferation. Hsa-miR-21 has been shown to downregulate tumor suppressor *PTEN* resulting in cell proliferation in cervical (25) and colorectal (26) cancers as well. Additionally, upon targeting the tumor suppressor *VHL* hsa-miR-21 has been

shown to promote the metastatic phenotype in cervical cancer (27). Most recently, co-delivery of gemcitabine and anti-miR-21 oligonucleotide resulted in synergistic antitumor effect in pancreatic cancer cells, paving the path for miR-21-targeted therapies (28).

The proto-oncogenic consequences of elevated expression of hsa-miR-106b has been observed in gastric cancer (29) and malignant melanoma (30), too. Additionally, the important cyclin-dependent kinase-inhibitor p21 has been confirmed to be a direct target of hsa-miR-106b (29, 30), while the downregulation of hsa-miR-106b promotes G1 phase arrest (30). Hsa-miR-10a has been found to be overexpressed in non-small-cell lung cancer (NSCLC) (31). Additionally, hsa-miR-10a has been shown to directly target the tumor suppressor gene *PTEN*, contributing to the promotion of a more aggressive biological phenotype observed in NSCLC (31).

The most important finding of our study is the prognostic relevance of hsa-miR-21, hsa-miR-106b and hsa-miR-10a expression in PanNENs, among which hsa-miR-21 has been shown to be an independent prognostic factor in the multivariate analysis in the largest cohort of PanNEN miRNA studies. Moreover, interestingly, in patients with disease progression, the higher expression of hsa-miR-10a and hsa-miR-106b, but not of hsa-miR-21 predicted earlier disease progression; however, neither of them was confirmed as an independent prognostic factor in this regard. In the next step, a prospective analysis of these miRNAs from body fluids upon different types of liquid biopsy in PanNEN patients may contribute to a more precise characterization of PanNENs before surgery providing new tools for precision oncology.

In conclusion, we detected and successfully validated the altered expression of hsa-miR-21, hsa-miR-106b and hsa-miR-10a on the largest PanNEN cohort for miRNA expression studies to date. Additionally, these miRNAs, especially hsa-miR-21, have prognostic relevance regarding progression-free and overall survival, which prompt us for further studies regarding its utilization in the early diagnosis of PanNENs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Author contribution statement

A P conceived the study. V K G and A P designed research. V K G performed *in silico* analysis. K B performed immunohistochemistry and histopathologically analyzed samples. V K G performed qRT-PCR measurements. V K G, A K, K B, P I and A P analyzed data. V K G drafted the manuscript. All authors acknowledged the final version of the manuscript.

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