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MiRNA-3653 Is a Potential Tissue Biomarker for Increased Metastatic Risk in Pancreatic Neuroendocrine Tumours

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Abstract

Pancreatic neuroendocrine tumours (PNETs) are relatively uncommon, accounting for 1–2% of all pancreatic neoplasms. Tumour grade (based on the Ki67 proliferative index and mitotic rate) is associated with metastatic risk across large cohorts; however, predicting the behaviour of individual tumours can be difficult. Therefore, any tool which could further stratify metastatic risk may be clinically beneficial. We sought to investigate microRNA (miRNA) expression as a marker of metastatic disease in PNETs. Tumours from 37 patients, comprising 23 with locoregional disease (L) and 14 with distant metastases (DM), underwent miRNA profiling. In total 506 miRNAs were differentially expressed between the L and DM groups, with four miRNAs (miR-3653 upregulated, and miR-4417, miR-574-3p and miR-664b-3p downregulated) showing statistical significance. A database search demonstrated that miRNA-3653 was associated with ATRX abnormalities. Mean survival between the two groups was correlated with mean expression of miRNA-3653; however, this did not reach statistical significance ($p = 0.204$). Although this is a small study, we conclude that miRNA-3653 upregulation may be associated with an increased risk of metastatic disease in PNETS, perhaps through interaction with ATRX and the alternate lengthening of telomeres pathway.

Keywords MicroRNA · Pancreatic neuroendocrine tumour · PNET

Introduction

Pancreatic neuroendocrine tumours (PNETs) comprise a heterogeneous group of tumours originating in the islet cells of the pancreas, accounting for 1–2% of primary pancreatic tumours [1, 2]. Recent data from the Surveillance, Epidemiology and End-Result (SEER) Program have reported a threefold increase in the reported incidence of PNETs from 0.17 to 0.43/100,000 person-years over the last three decade since 1970 [1, 3]. PNETs are classified as either functioning (F-PNETs) or non-functioning (NF-PNETs) based upon whether they cause a clinical syndrome as a result of hormone secretion [4]. In general, functional PNETs tend to present earlier due to symptoms, while NF-PNETs often are asymptomatic in early stages, presenting later and as a result of tumour mass effect [5].

Surgical resection plays a central role in the treatment of most PNETs, conferring a significant survival advantage

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[6]. Aggressive locoregional resection as well as resection of metastases is indicated and can achieve cure. Debulking surgery is effective in patients with metastatic disease to achieve symptom control and survival prolongation [7, 8]. The American National Cancer Database (NCD) have reported 5-year survival rates at 61%, 52%, 41% and 16% for stages I, II, III and IV for PNETs respectively [9].

Whilst genetic aberration account for the pathogenetic basis of syndrome-associated PNET (i.e. those associated with multiple endocrine neoplasia type 1 (MEN1), von Hippel Lindau (VHL), neurofibromatosis type 1 (NF1), tuberous sclerosis complex or glucagon cell adenomatosis (GCA)), the mechanisms by which sporadic PNETs develop are still poorly understood. Dysregulation of microRNAs, short non-coding RNAs ~ 17–25 nucleotides in length, has been shown to play an important role in the development of many cancers through their post-transcriptional regulation of gene expression [10, 11]. Few studies to date, however, have investigated the role of miRNAs as diagnostic or prognostic markers in PNETs [12–14]. The aim of the present study was to contribute to the knowledge of microRNA profiles of PNETs and identify candidate miRNAs that correlated with the presence (at diagnosis) or development of metastatic disease.

Methods

Patient Selection

All patients who underwent resection of pancreatic neuroendocrine tumours at our facility between 1992 and 2014 were retrospectively included in this study. Patients were divided into two groups based on whether their disease was locoregional only (L) or whether they had distant metastases either at the time of surgery or during their postoperative follow-up (DM). Pathological data was collected from an established surgical pathology database and clinical data was collected from patient records kept through rigorous outpatient follow-up and surveillance. All patients had resection with curative intent including enucleation (insulinoma only), pancreatic resection ± regional lymph node clearance ± limited liver resection for isolated metastasis. The local Human Research Ethics Committee approved the study.

Tissue Samples

PNET specimens were obtained from archived formalin-fixed, paraffin-embedded (FFPE) blocks. For the purpose of this study, 5 × 5 μ shavings from each specimen were harvested from areas containing > 90% tumour cells.

RNA Extraction

The FFPE RNeasy Kit (Qiagen, Hilden, Germany) was used for the extraction of total RNA according to the manufacturer's instructions.

RNA Quality Assessment

A Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, Waltham, Mass) was used to measure concentrations (A260), protein contamination (A260:A280 ratio) and contamination with organic compounds or buffer components (A260:A230 ratio) in extracted RNA samples.

MiRNA Microarray

MiRNA microarray profiling was performed by Exiqon Services (Vedbaek, Denmark). Total RNA quality was first determined using an Agilent 2100 Bioanalyzer profile (Agilent, USA). The miRCURY LNA microRNA Hi-Power Labeling Kit, Hy3TM/Hy5TM (Exiqon, Denmark) was used to label 150 ng each of total RNA from each sample with Hy3TM and reference with Hy5TM fluorescent label according to the manufacturer's instructions. Labelled samples were mixed in a pair-wise fashion before hybridization to the miRCURY LNA microRNA Array 7th Generation (Exiqon, Denmark) as per the manufacturer's instruction manual using a Tecan HS4800 hybridization station (Tecan, Austria). This targeted all microRNAs for human, mouse and rat registered in the miRBASE 18.0 and contains 3100 capture probes. Microarray slides were then scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) in an ozone-free environment. ImaGene 9.0 software (BioDiscovery, Inc., USA) was used for image analysis. Quantified signals were background corrected and normalised using the quantile normalisation method.

MicroRNA Candidate Selection/Statistical Analysis

For selection of potential microRNA candidates, mean log median ratios (LMRs), differences in LMRs and associated p values between groups of samples were determined. MiRNAs of potential significance were identified by a fold change of greater than or equal to two and p value < 0.05. All calculations for the microarray component of the study were performed by Exiqon in the software R/Bioconductor (mainly using the limma package). All other statistical analysis including the comparison of clinical characteristics using chi-squared and students t test as well as Kaplan-Meier survival analysis was performed using SPSS version 24 (IBM Corporation, NY, USA).

Prediction of MicroRNA Targets

Bioinformatics tools were used to predict putative gene targets for miRNAs differentially expressed to a statistically significant degree. Several databases were used to minimise disconcordance and identify genuine miRNA targets. These included TargetScan, MiRBase and TarBase. Target genes identified as targets across all three databases (for each miRNA) were identified. Those targets associated with known PNET pathogenesis pathways were shortlisted.

Results

Clinicopathologic Characteristics

Thirty-seven patients (19 females and 18 males) were included in the present study (see Table 1). Twenty-three patients had locoregional disease only. Four patients had solitary liver metastases at initial presentation while 10 developed distant metastases during the course of their follow-up (see Table 2).

Table 1 Summary of patient characteristics

	Distant metastases	Locoregional	*p* value
Mean age (range)	55.9 (32.8–79.0)	62.9 (41.0–87.1)	0.167
Sex			
Female	7	12	
Male	7	11	0.898
Aetiology			
Sporadic	14	20	
Syndromic (MEN1)	0	3	0.159
Type			
Functional	3	11	
Non-functional	11	12	0.108
Subtype			
NFPNET	11	12	
Insulinoma	2	6	
Gastrinoma	0	5	
Glucagonoma	1	0	0.101
Perineural invasion			
Absent	11	23	
Present	3	0	0.021
Vascular invasion			
Absent	5	19	
Present	9	4	0.004
WHO 2010 Grade			
1	6	16	
2	7	7	
3	1	0	0.168

Table 2 Location of metastases in DM group

Site	*n*
Liver only	9
Liver and chest	2
Liver, mediastinum and bone	1
Liver and lymph node	1
Liver and bone	1

Across all patients, mean age at resection was 60.3 years. Mean age at time of resection for patients who had or went on to develop distant metastases was 55.9 years. Patients with only locoregional disease tended to be older (62.9 v 55.9 years; $p = 0.167$). The majority of patients had sporadic PNETs (34 patients; 92%), whilst 3 patients (8%) had PNETs associated with MEN1 syndrome. No difference between groups existed based upon sporadic versus syndromic tumours. Similarly, there was no difference between the two patient groups based on tumour type (functional versus non-functional) or subtype (NF-PNET, insulinoma, gastrinoma or glucagonoma). Perineural invasion (21% v 0%; $p = 0.021$) and vascular invasion (64% v 17%; $p = 0.004$) were observed to be more common in patients who developed distant metastases.

Survival Analysis

Patients in the subgroup DM had poorer overall survival (OS) as compared with subgroup L ($p = 0.046$) (Fig. 1).

Microarray Analysis

MicroRNA profiling microarray was performed on the tumours of all 37 patients. In total, 506 miRNAs with differential expression between the 'distant metastasis' group and 'locoregional' group were identified. Two hundred sixty-five of these miRNAs were downregulated, whilst 241 were upregulated. Of these, 4 miRNAs were differentially expressed to statistical significance. These included miR-3653 which was upregulated and miR-4417, miR-574-3p and miR-664b-3p which were all downregulated. Fold change and *p* values for these miRNAs are summarised in Table 3.

Mean expression of miR-3653 in the locoregional group was 5.85 and in the distant metastasis group was 6.30, that is a greater degree of upregulation. Student's *t* test revealed that this difference was statistically significant ($p = 0.024$).

MicroRNA Targets/In Silico Analysis

The four potential candidate miRNAs with significantly differential expression between DM and L groups were further examined/investigated in terms of potential role in PNETs pathogenesis. Only miRNA-3653 was identified by all three

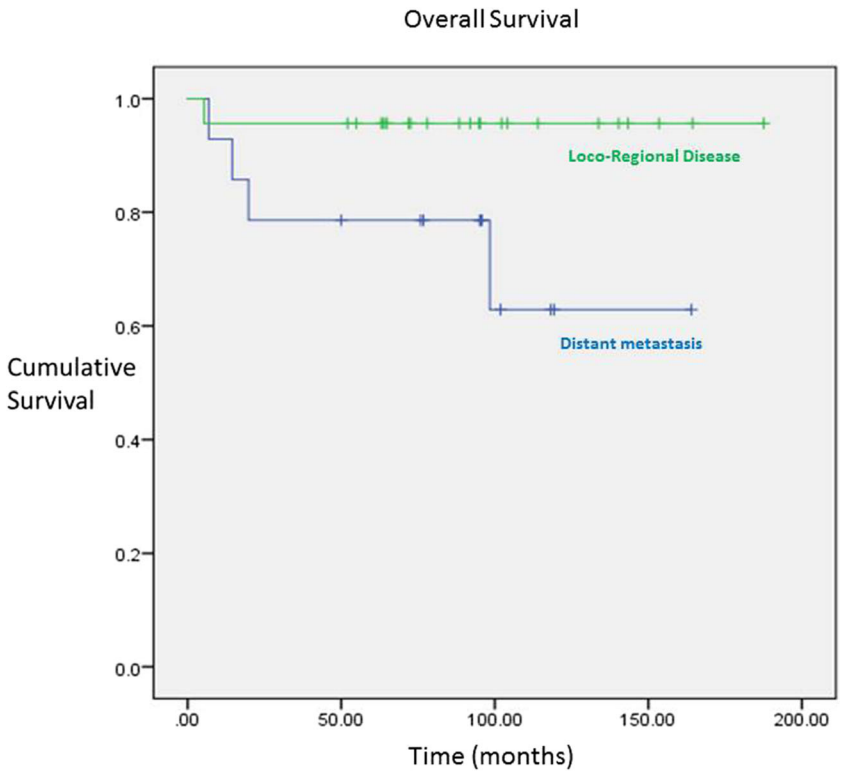

Fig. 1 Kaplan-Meier analysis comparing overall survival in patient groups

databases as having a potential PNET-related target, ATRX. Other miRNAs with significantly differential expression had no targets related to current PNET pathways or were not present across the three databases used.

Correlation Between miR-3653 and Overall Patient Survival

Mean survival between the two groups was correlated with mean expression of miRNA-3653; however, this did not reach statistical significance ($p = 0.204$).

Discussion

The purpose of this study was to identify miRNAs that could potentially serve as prognostic biomarkers in PNETs. In our patient cohort, as expected, overall survival in patients with distant metastases was significantly shorter than patients with locoregional disease only and we identified several significantly differentially expressed microRNAs between these two patient groups. Of these differentially expressed miRNAs, only MiR-3653 expression, which was upregulated in the distant metastasis group, had a potential target gene that has been implicated in the pathogenesis of PNETs. Furthermore, this target was predicted across all three target-gene databases searched. These findings suggest that miR-3653 may be an indicator of a subset of patients with tumours of a more biologically aggressive nature and therefore could serve as a biomarker for the development of metastatic disease, which in turn is associated with poorer prognosis.

Somatic mutations in the ATRX gene have been implicated in the development and progression of a variety of neoplasms including PNETs, osteosarcomas, malignant phaeochromocytoma, gliomas and astrocytomas [15–19]. This gene encodes a protein of the same name that acts as a transcription regulator and is a member of the SWI/SNF family of chromatin remodelling proteins [20]. ATRX protein is thought to play a role in chromatin stabilisation and remodelling by binding to histone H3. Forming a complex with death-associated protein 6 (DAXX), the ATRX-DAXX dimer recruits histone methyltransferase SUV39H which methylates lysine 9 and maintains chromatin in a state of repression [21]. It has also been shown

Table 3 Fold change and p values for microRNAs with significant differential expression between DM and L groups

MicroRNA	Fold change (log)	p value
miR-3653	0.445	0.0207
miR-4417	− 0.442	0.0430
miR-574-3p	− 0.377	0.0164
miR-664b-3p	− 0.252	0.0342

to be involved in gene regulation at the interphase stage as well as in the segregation of chromosomes during mitosis [22].

Patients with mutations in either ATRX or DAXX have been shown to exhibit the alternative lengthening of telomeres (ALT) phenotype [21, 23], in which telomere stabilisation facilitates tumour progression by preventing their shortening to a critical size at which apoptosis would otherwise ensue [24]. A recent study correlated DAXX/ATRX mutations and ALT activation with chromosomal instability in patients with PNETs. This study also found that DAXX/ATRX mutations and chromosomal instability were associated with larger primary tumours and late stage tumours, suggesting factors are important at later stage as a 'transforming' (as opposed to an initiating) changes in tumorigenesis [24]. Inactivating mutations in ATRX or DAXX have been found in 43% of patients with sporadic PNETs in a series of 68 patients [25]. This study, which included only grade 1 and 2 PNETS, reported mutation in the MEN1 gene in 43% of patients and mTOR pathway mutations in 14% of patients [25]. Given that ATRX is a predicted gene target of miR-3653, upregulation of miR-3653 in patients with distant metastases and associated poorer overall survival seen in our study could act through this mechanism. ATRX and DAXX mutations were found in only 6% of patients with MEN1 PNETs in another series [26].

To date, there are limited studies of miRNAs in PNETs. The majority of these studies examined differential expression of miRNAs between PNETs and normal pancreatic tissue. MiR-193b expression has been reported as a discriminator of PNETs in both tissue and serum from normal pancreatic tissue [13]. Over-expression of miR-103 and miR-107 and downregulation of miR-155 have also been reported in PNET tissue compared with normal tissue [12]. Data on the association between miRNAs and biological behaviour of PNETs is lacking. Lee et al. [14] reported that high miR-196a expression was significantly correlated with advanced T stage, higher ki-67% index and higher mitotic counts. Furthermore, higher miR-196a levels were associated with decreased disease-free and overall survival. Another study demonstrated a strong association between an increased miR-21 expression and higher ki67 index as well as the development of liver metastases [12]. Finally, miR-210 has been shown to correlate with metastatic disease and miR-642 [13]. To our knowledge, the present study is the first to report an association between miR-3653 and PNETs. A review of the literature performed on PubMed found only two other publications citing miR-3653. The first, by Lin et al. [27], found that miR-3653 was an independent predictor of both recurrence-free survival and overall survival in a series of lung adenocarcinoma tissue specimens. The other reported that MiR-3653 was upregulated in cervical cancer associated with human papillomavirus (HPV) compared with normal tissue [28]. To our knowledge, this is the first study identifying miR-3653 as miRNA of potential significance in PNETs. It is also the first instance where ATRX has been discussed in the context of being a predicted target of miRNA-3653.

The current study is limited by a small sample size where the majority of patients (all except one) had low-grade (WHO grade1/2) tumours limiting a wider analysis of the different biological spectrum of PNETs. We did not separately analyse the patients with known MEN1 syndrome due to their limited number ($n = 3$). Whether miR-3653 expression correlates with tumour grade and the way in which tumour grade correlates with patient survival across all grades of PNET cannot be examined in our study. Furthermore, only tumour tissue was examined, thus relative expression of miRNAs between PNETs in different patients has been analysed rather than change in expression compared each individual patient's 'normal' tissue. Finally, identified miRNAs with differential expression are yet to be validated. Further work is also needed to examine in more detail the effect relationship between miR-3653 and its suspected target gene ATRX.

In conclusion, the present study identified a higher expression of tumour miR-3653 in PNET patients who developed metastatic disease following surgical resection, providing an impetus to suggest that this may become a candidate marker of invasion and metastasis thereby heralding an overall poorer prognosis. Several bioinformatics tools have predicted transcriptional regulator ATRX as a possible target for miR-3653 and this may be mediated by inactivation of ATRX.

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Compliance with Ethical Standards

Conflict of Interest Author Preetoje Gill declares that she has received a research grant from IPSEN which supported this study.

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