

# Downregulation of the let-7 family of microRNAs may promote insulin receptor/insulin-like growth factor signalling pathways in pancreatic ductal adenocarcinoma

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**Abstract.** Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer type characterized by dysregulated cell signalling pathways and resistance to treatment. The insulin-like growth factor (IGF) signalling pathway has been identified to have a role in tumour progression and therapy resistance. However, its regulatory roles in PDAC have remained to be fully elucidated. In the present study, dysregulated microRNAs (miRNAs) in PDAC were explored with a focus on those that may be involved in regulating the insulin/IGF signalling pathway. A total of 208 patients were recruited, comprising 112 patients with PDAC, 50 patients with chronic pancreatitis (CP) and 46 subjects as a control group (CG). miRNA-specific quantitative PCR assays were used to measure 300 candidate miRNAs. The Student's t-test was applied to compare miRNA regulation between cancer patients and controls with a false discovery rate correction using Bonferroni-type comparison procedures. The DIANA-mirPath v.3 tool and HMDD v3.0 were used to identify miRNA-mRNA interactions within specific pathways. In patients with PDAC, 42 miRNAs were significantly upregulated and 42 were downregulated compared to the CG ( $P < 0.01$ ). In the PDAC vs. CP analysis, 16 significantly ( $P < 0.01$ ) upregulated and 16 downregulated miRNAs were identified. Of note, members of the let-7 family of miRNAs were downregulated and were indicated to target several components of the insulin receptor (INSR)/IGF pathway, including receptors and binding proteins, for upregulation and thus, may enable the activation of the pathway. Downregulation of the let-7 family may help promote the INSR/IGF pathway in PDAC. It

may thus be an effective target for the development of INSR/IGF pathway-specific treatment strategies.

## Introduction

According to 2018 GLOBOCAN statistics, pancreatic cancer had 458,918 new cases and 432,242 mortalities, which accounted for 4.5% of cancer-associated deaths worldwide (1,2). Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer, has a poor prognosis with a five-year overall survival rate of 5%, which has not significantly improved over the last two decades despite improved chemotherapeutic and biological agents (3). Hence, there is a requirement to better understand the underlying molecular mechanisms of the disease as a basis for identifying novel and more effective therapeutic targets.

More than one growth factor potentiates carcinogenesis and multiple growth factor signalling pathways are consequently upregulated in cancerous cells (4-6). The epidermal growth factor family of receptors (EGFR) are overexpressed in >60% of PDAC tumours (7); however, EGFR-targeted therapies have been unsuccessful in improving overall patient survival (5,8). Hence, there must be a simultaneous upregulation of alternative pathways. One such potential pathway is the insulin/insulin-like growth factor (IGF) pathway. In PDAC, overexpression of both IGF-1 and type 1 IGF receptor (IGF-1R) has been demonstrated (9). Furthermore, EGFR has been identified to be co-expressed with IGF-1R, an interaction that confers resistance to EGFR-specific therapies (10).

The IGF signalling pathway is important for cellular growth, proliferation and differentiation, as well as for tissue integrity (11). It has been indicated to be aberrant in PDAC (12), enhancing tumorigenesis and therapeutic resistance (13); furthermore, IGF-1R is associated with increased proliferation and angiogenesis and decreased apoptosis (14-16). A recent study by our group reported on the upregulation of the IGF signalling pathway in resected pancreatic tumours obtained from South African patients (17). Furthermore, IGF-1R has been demonstrated to have predictive and prognostic value, as its overexpression has been associated with metastases and decreased overall survival in PDAC (5), highlighting

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the requirement to further investigate the regulation of this pathway and its mechanism of action in PDAC.

MicroRNAs (miRNAs) are non-coding RNAs ~17-24 nucleotides in length, which are involved in biological and cellular processes by regulation of mRNA translation (18,19). Circulating miRNAs have been determined to be involved in tumorigenesis serving as biomarkers and therapeutic targets (20-24). In the present study, differentially expressed miRNAs in patients with PDAC compared to patients with chronic pancreatitis (CP) and a control group (CG) were identified. Using bioinformatics analysis, the possible regulation of the insulin receptor (INSR)/IGF signalling pathway activity by miRNAs was further investigated.

## Patients and methods

**Patients.** A total of 112 patients with cytologically or histologically confirmed treatment-naïve PDAC located in the head of the pancreas were recruited at the time of diagnosis in Chris Hani Baragwanath Hospital, Johannesburg, South Africa from January 2014 to December 2016. Participants were categorized into three clinical groups according to their TNM staging (25). Group 1 consisted of participants with resectable, non-metastatic disease (TNM stage 0-IIB), Group 2 comprised patients with non-resectable disease without evidence of metastases (TNM stage III) and Group 3 included participants with metastatic disease (TNM stage IV). Participants with tumours other than PDAC were excluded. All clinical information was collected and stored using REDCap v6.7 electronic data capture tools (26).

As the CP group, patients who had completed at least one year of out-patient follow-up with the Hepatopancreatobiliary unit in Chris Hani Baragwanath Hospital, (Johannesburg, South Africa) and admitted between January 2014 to December 2016 and had an abdominal CT scan demonstrating no mass or lesion suspicious of a tumour in their pancreas within two weeks of their recruitment date were included. CP was diagnosed radiologically or endoscopically according to international guidelines (27,28). Any patient with CP and a pancreas mass or cyst was excluded.

As the CG, consecutive patients admitted to the vascular ward in Chris Hani Baragwanath Hospital (Johannesburg, South Africa) and admitted between January 2014 to December 2016 with severe peripheral arterial disease who underwent an abdominal CT scan as part of their clinical work-up were included if the scan demonstrated a healthy pancreas. Participants were excluded if they had a history of any previous malignancy or pancreatic disease, obstructive jaundice, unexplained diarrhoea or weight loss.

**Sample processing.** From each participant, venous blood samples were collected in 4.5-ml tubes containing EDTA and stored at room temperature. Samples were centrifuged for 15 min at 1,500 x g at room temperature within 4 h of collection. The plasma was aliquoted into 1-ml Eppendorf tubes and stored at -80°C. Analysis was performed within 12 months of sample collection and storage.

**Reverse transcription-quantitative PCR (RT-qPCR).** Plasma miRNA was extracted using the miRNeasy serum/plasma

miRNA isolation kit (Qiagen GmbH). The ID3EAL miRNA knowledge panel 384 Target kit (MiRXES) was used and experiments were performed according to the manufacturer's instructions. The kit contained the ID3EAL Panel RT Primer Pool for the miRNAs with a set of three proprietary spike-in RNAs [ID3EAL Panel RNA Spike-In (MiRXES)] to normalize variations in RNA isolation efficiency. Isolated serum RNA was subjected to RT using the ID3EAL Reverse Transcriptase kit (MiRXES). A 6-log serial dilution of synthetic templates for each miRNA was concurrently reversed-transcribed. Using the miRNA-specific qPCR assays-ID3EAL miRNA qPCR Master Mix (MiRXES), >300 candidate miRNAs were measured in each complementary DNA sample. The following cycling conditions were used: 95°C for 10 min, 40°C for 5 min, and then 45 cycles of 95°C for 10 sec and 60°C for 30 sec. Absolute copy numbers of each miRNA were determined by interpolation of the C<sub>q</sub> values to that of the synthetic miRNA standard curves and adjusted for RT-qPCR efficiency variation. The  $2^{-\Delta\Delta C_q}$  method was used for quantification (29).

**Data normalization and statistical analysis.** A set of three endogenous reference miRNAs were identified using geNorm and NormFinder (30,31). The geometric mean of the three normalizers was used to normalize the miRNA expression data across subjects. Student's t-test was applied to compare miRNA expression between cancer and control groups, with false discovery rate correction using Bonferroni-type comparison procedures (32). The Sequential Forward Floating Search algorithm (33) was used to optimize the biomarker selection during the internal cross-validation process for the discovery study and AUC values were set as the optimization target. The linear support vector machine was used to construct multi-variant biomarker panels with optimal performance in classifying control and cancer groups. Demographic data and clinical characteristics of the three patient groups were compared using one-way analysis of variance. Bonferroni's test was used for post hoc analysis. P<0.05 was considered to indicate statistical significance. The Stata v14 statistical programme (StataCorp) was used for analyses and values expressed as n (%) or median (range). For values represented as n (%), a student's t-test was performed for the normally distributed continuous variables, and P<0.05 was considered significant.

**Bioinformatics analysis.** Dysregulated miRNAs were considered if they had a significance of P<0.01 and absolute fold change of >1.5. The DIANA-mirPath v.3 tool was used for interaction and pathway analyses (34). The tool uses Tarbase v.8 to identify miRNA-mRNA interactions and probes the Kyoto Encyclopedia of Genes and Genomes for pathway analysis (35,36). GeneMania and HMDD v3.0 were used to visualize interactions of target genes and observe miRNA-interacting partners, respectively (37,38). The highlighting of key genes of interest on pathways and network was drawn using Microsoft PowerPoint v2013 (Microsoft).

## Results

**Clinicopathological characteristics of study participants.** The study included 208 subjects, comprising 112 patients with PDAC, 50 patients with CP and 46 patients with critical limb

Table I. Demographic and clinical characteristics of patients recruited for the study.

Variable	PDAC (n=112)	CP (n=50)	Control (n=46)	P-value (PDAC vs. CP)	P-value (PDAC vs. control)
Male/female ratio	1.65:1	4.2:1	1.4:1	0.179	0.901
Age <sup>a</sup> , years	59 (33-85)	52 (38-69)	61 (35-89)	P<0.001	0.704
Chronic <sup>b</sup> illness	69 (62)	16 (32)	31 (67.3)	0.095	0.692
Smoking status <sup>b</sup>					
Ever smoked	72 (64)	44 (88)	26 (57.1)	P<0.003	0.120
Current smoking	29 (26)	34 (68)	7 (15)	P<0.001	P<0.02
Alcohol use <sup>b</sup>	78 (70)	45 (90)	23 (50)	P<0.004	0.088
Body mass index <sup>a</sup> , kg/m <sup>2</sup>	21.4 (15.6-29.2)	19.0 (14.8-24.5)	22.1 (16.1-26.3)	0.366	0.423

Values are expressed as <sup>a</sup>median (range) <sup>b</sup>n (%). PDAC, pancreatic ductal adenocarcinoma; CP, chronic pancreatitis.

ischaemia as a CG. The demographic features and traditional risk factors of the subjects are provided in Table I. In the PDAC group, there were 15 patients in Group 1, 57 patients in Group 2 and 40 in Group 3. The 6-month overall survival rate for Groups 1, 2 and 3 was 80.0, 91.2, and 32.5%, respectively (Table SI). There were 35 patients with survival for <6 months, 27 of which were from Group 3. The body mass index of all of the patients was determined and was not significantly different between the three study groups.

**Differential expression of miRNA.** miRNA expression was compared between the PDAC group and the CG and 42 significantly upregulated and 42 downregulated miRNAs were identified (P<0.01; Table SII). When comparing the PDAC group to both the control and chronic pancreatitis groups combined together (PDAC vs. CG+CP), 16 significantly upregulated and 16 downregulated miRNAs in PDAC were identified (P<0.01; Fig. 1; Table SIII). Of note, the downregulated miRNAs included members of the let-7 family, including *Homo sapiens* (hsa)-let-7f-5p and hsa-let-7a-5p. Comparison of the PDAC group with solely the CP group provided only three significantly upregulated and four downregulated miRNAs (P<0.01; Table SIV).

**Let-7 miRNAs target the INSR and IGF signalling pathways.** Using a targeted bioinformatics analysis, it was demonstrated that the downregulated and upregulated miRNAs targeted several components of the insulin pathway. However, only the downregulated miRNAs were observed to target INSR (Figs. 2 and 3). Amongst the downregulated miRNAs, members of the let-7 family, hsa-let-7f-5p and hsa-let-7a-5p, were implicated in targeting INSR.

Furthermore, the HDMM tool was used to illustrate the interaction between the let-7 family of miRNAs with several genes, including members of the IGF signalling pathway (Fig. 3). The analysis suggested that let-7 targets the IGF signalling pathway for downregulation. Let-7 has an inhibitory effect on the IGF pathway and is downregulated, suggesting the subsequent upregulation of this pathway.

**Expression of the let-7 family in different stages of PDAC.** The levels of let-7 miRNAs were differentially expressed in

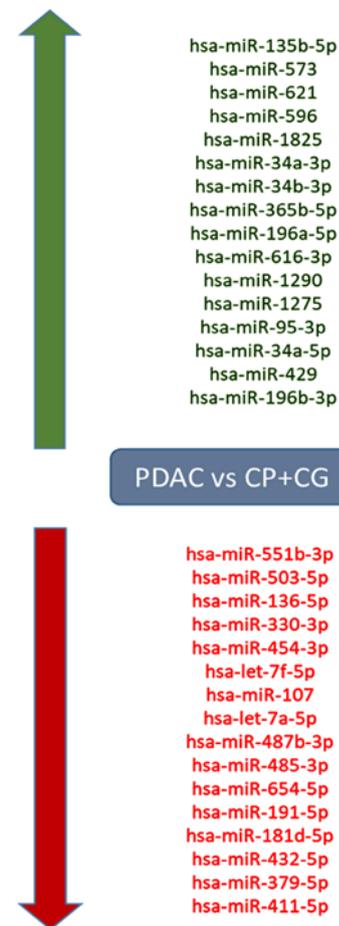


Figure 1. Dysregulated miRNAs in patients with PDAC. Compared to patients with CP and the control group, patients with PDAC had 16 significantly upregulated and 16 downregulated miRNAs (P<0.01). The red colour indicates downregulation and green denotes upregulation. CP, chronic pancreatitis; CG, control group; PDAC, pancreatic ductal adenocarcinoma; hsa, *Homo sapiens*; miRNA/miR, microRNA.

different groups of PDAC (Table SVa). Further analysis across the PDAC groups indicated that 5 miRNAs were significantly upregulated in Group 3 compared to Groups 1 and 2 combined (P<0.05; Table SVb). Although the differences in expression levels were not significant between ‘Group 3 vs. 1+2’, certain



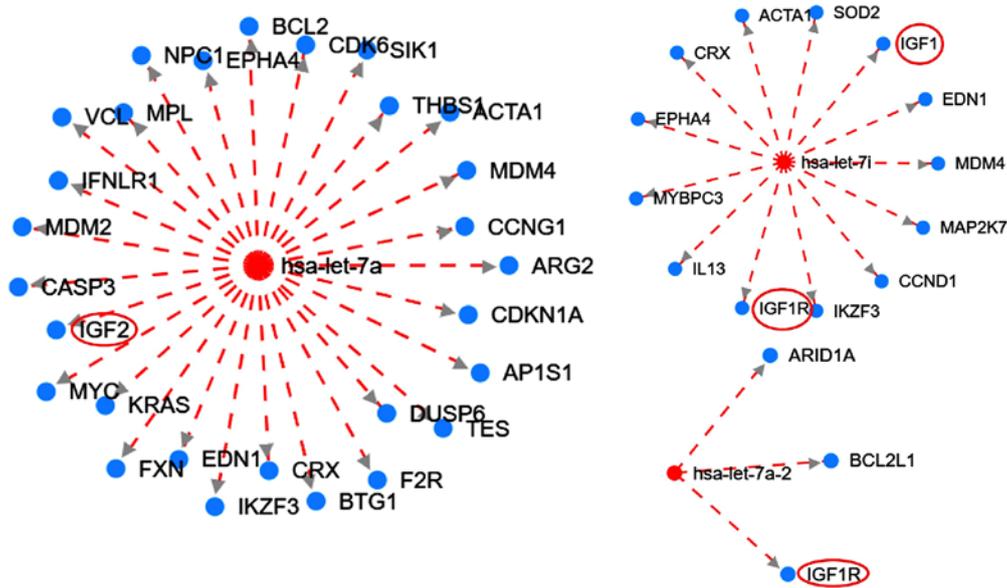


Figure 3. Interaction network of the let-7 family targeting the IGF family members generated from HMDD version 3. Red circles (added with Microsoft PowerPoint v2013) indicate members of the IGF signaling pathway. The broken red lines indicate downregulation. Red and blue nodes are microRNAs and genes, respectively. IGF1R, type 1 insulin-like growth factor receptor; IGF, insulin-like growth factor; ARID1A, AT-rich interaction domain 1A; BCL2L1, BCL2 like 1; EDN1, endothelin 1; MDM4, MDM4 regulator of p53; MAP2K7, mitogen-activated protein kinase kinase 7; CCND1, cyclin D1; IKZF3, IKAROS family zinc finger 3; IL13, interleukin 13; MYBPC3, myosin binding protein C3; EPHA4, EPH receptor A4; CRX, cone-rod homeobox; ACTA1, actin  $\alpha$ 1 skeletal muscle; SOD2, superoxide dismutase 2; BCL2, BCL2 apoptosis regulator; CDK6, cyclin dependent kinase 6; SIK1, salt inducible kinase 1; THBS1, thrombospondin 1; CCNG1, cyclin G1; ARG2, arginase 2; CDKN1A, cyclin dependent kinase inhibitor 1A; AP1S1, adaptor related protein complex 1 subunit  $\sigma$ 1; DUSP6, dual specificity phosphatase 6; TES, testin LIM domain protein; F2R, coagulation factor II thrombin receptor; BTG1, BTG anti-proliferation factor 1; FXN, frataxin; KRAS, KRAS proto-oncogene; MYC, MYC proto-oncogene; CASP3, caspase 3; MDM2, MDM2 proto-oncogene; IFNLR1, interferon  $\lambda$  receptor 1; VCL, vinculin; MPL, MPL proto-oncogene; EPHA4, EPH receptor A4; hsa, *Homo sapiens*.

members of the let-7 miRNAs were differentially expressed across the various groups. When comparing Group 3 to a combination of Groups 1 and 2, hsa-let-7b-5p, hsa-let-7a-3p and hsa-let-7d-3p were upregulated and hsa-let-7a-5p and hsa-let-7f-5p were downregulated. Furthermore, hsa-let-7b-3p, hsa-let-7d-3p, hsa-let-7a-5p and hsa-let-7e-3p were upregulated in Group 3 vs. Group 2 but downregulated in Group 2 vs. 1. On the other hand, hsa-let-7a-5p and hsa-let-7f-5p were downregulated in Group 3 vs. 2 but upregulated in Group 2 vs. 1. The potential prognostic value of the let-7 miRNAs was also assessed by comparing levels observed in patients with a ‘poor prognosis’ and those with a ‘good prognosis’ (Table SI). A poor or good prognosis was defined by the survival of patients in months; and since patients were followed up for only one year, those with a survival time of <12 months were categorized as having a ‘poor prognosis’. Comparing patients in the poor vs. good prognosis group, a total of 20 miRNAs were associated with poor prognosis, as they were significantly upregulated in the poor prognosis group ( $P < 0.05$ ). However, none of the let-7 family members was significantly dysregulated (Table SVI).

**Discussion**

In the present study, the number of dysregulated miRNAs in patients with PDAC compared with the CG was higher than that in the PDAC compared with the CP group. The decreased number of significantly dysregulated miRNAs between the CP and the PDAC group may be due to shared pathological molecular mechanisms, as CP is a risk factor for the development of PDAC (39). The present study indicated that members

of the let-7 miRNA family are dysregulated in PDAC. Of note, the let-7 family members were significantly downregulated in patients with PDAC. Let-7a-5p and let-7f-5p were also downregulated in the late stages of PDAC. This finding corroborates the observation that downregulation of let-7 in gemcitabine-resistant pancreatic cancer cells was associated with activation of the epithelial-to-mesenchymal pathway characteristic of metastasis (40).

The let-7 family inhibits the expression of components of the INSR/IGF signalling pathway (Figs. 2 and 3) and their downregulation may result in increased activity of this pathway. This suggests that hypothetically, an increase in let-7 may inhibit the INSR/IGF signalling pathway and subsequently inhibit cellular proliferation and promote chemotherapeutic sensitivity (Fig. 4). The let-7 family was initially identified in the nematode *Caenorhabditis elegans*. In humans, the let-7 family has 13 members, which have been demonstrated to have a significant role in the process of carcinogenesis (41). In 2016, Encarnación *et al* (42) indicated that overexpression of let-7b in patients with breast cancer resulted in increased DNA repair capacity. Aberrant DNA repair capacity is characteristic of PDAC (43,44). Previous studies have suggested that expression of the let-7 family was significantly reduced in PDAC tumor cells as compared with normal acinar cells and that induction of let-7 expression inhibited cellular proliferation (45). One study indicated that treatment with diflourinated curcumin, a curcumin analogue with anti-oxidant properties, inhibited tumor growth and this was associated with increased levels of let-7 (46).

Early-onset diabetes and obesity are risk factors for PDAC (47). It is known that obesity-linked upregulation of the



resistance (61). Therefore, upregulation of let-7 may inhibit stromal formation, increasing tumor chemosensitivity.

In conclusion, the present study demonstrated down-regulation of certain members of the let-7 family in plasma samples of patients with PDAC compared to patients with CP, a pre-malignant condition, as well as to a CG with significant risk factors common to patients with PDAC. Since the *in silico* analysis indicated that let-7 targets the INSR/IGF pathway, its downregulation may increase the expression of the INSR/IGF pathway and may therefore be an effective target for the development of INSR/IGF pathway-specific treatment strategies. Future studies investigating the effects of let-7 in modulating the IGF pathway in pancreatic cancer by using *in vitro* and *in vivo* systems should be performed to validate this hypothesis. Additionally, it would be pertinent to conduct a longer follow-up Kaplan Meier analyses for overall survival between patients that have high or low let-7 expression.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

MB was responsible for the conceptualization of the study, drafted and revised the manuscript. EEN performed data analyses, drafted and revised the manuscript. Both authors read and approved the final manuscript.

### Ethics approval and informed consent to participate

A prospective study including black South African participants was undertaken. Ethics approval was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical), certificate no. M130551. All patients recruited for the study provided written informed consent prior to sample collection.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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