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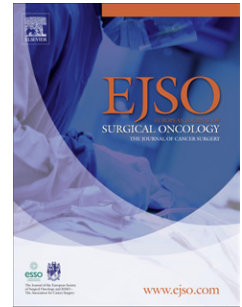
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Authors: KH Chang^a, N Miller^a, EAH Kheirelseid^a, H Ingoldsby^b, E Hennessy^a, CE Curran^a, S Curran^b, MJ Smith^a, M Regan^a, OJ McAnena^a, MJ Kerin^a

Affiliation: ^a Department of Surgery, National University of Ireland, Galway,
Republic of Ireland

^b Department of Pathology, National University of Ireland, Galway,
Republic of Ireland

Corresponding author: Dr. Nicola Miller

Address: Department of Surgery,
National University of Ireland, Galway,
Clinical Science Institute, Galway,
Ireland.

Email: nicola.miller@nuigalway.ie

Tel: +353 91 544637

Fax: +353 91 494509

Abstract

Introduction

MiRNAs regulate gene expression by binding to target sites and initiating translational repression and/or mRNA degradation. Studies have shown that *miR-21* exerts its oncogenic activity by targeting the *PDCD4* tumour suppressor 3'-UTR. However, the mechanism of this regulation is poorly understood. In colorectal cancer, loss of *PDCD4* has been reported in association with increased tumour aggressiveness and poor prognosis. The purpose of this study was to delineate the interaction between *PDCD4* and its oncogenic modulator *miR-21* in colorectal cancer.

Methods

A cohort of 48 colorectal tumours, 61 normal tissues and 7 polyps were profiled for *miR-21* and *PDCD4* gene expression. A subset of 48 specimens (31 tumours and 17 normal tissues) were analysed for PDCD4-protein expression by immunohistochemistry.

Results

A significant inverse relationship between *miR-21* and *PDCD4* gene expression ($p < 0.001$) was identified by RT-qPCR. In addition, significant reduction of *PDCD4* ($p < 0.001$) expression and reciprocal upregulation of *miR-21* ($p = 0.005$) in a progressive manner from tumour-polyp-normal mucosae was identified. Analysis of protein expression by IHC revealed loss of PDCD4 staining in tumour tissue. Patients with disease recurrence had higher levels of *miR-21*.

Conclusion

This study demonstrates the inverse relationship between *miR-21* and *PDCD4*, thus suggesting that *miR-21* post-transcriptionally modulates *PDCD4* via mRNA degradation. Pharmacological manipulation of the *miR-21/PDCD4* axis could represent a novel therapeutic strategy in the treatment of colorectal cancer.

Keywords: Colorectal cancer, PDCD4 tumour suppressor, miRNA-21

Introduction

Colorectal cancer (CRC) is the fourth commonest cancer in men and the third in women worldwide [1]. Despite advances in surgery and other treatment modalities, CRC remains a significant health care burden resulting in approximately 630,000 deaths globally each year. Pioneering work in CRC has led to the concept of adenoma-carcinoma sequence, which describes the stepwise progression of normal mucosa to adenoma to carcinoma with the accumulation of multiple genetic alterations [2]. Novel molecules and pathways involved in colorectal carcinogenesis continue to emerge in the search for improved targeted therapeutic strategies.

Mi(cro)RNAs are naturally occurring short RNA molecules that post-transcriptionally regulate gene expression by binding to the 3'-UTR of their target mRNAs. Antisense binding of a miRNA to its regulatory target with partial complementarity results in miRNA-RISC complex-induced translational repression [3]. Binding with perfect or near perfect complementarity between a miRNA and the 3'-UTR of its target mRNA, on the other hand, results in mRNA degradation through a distinct RNA interference pathway [4]. MiRNAs play major roles in governing diverse biological processes such as differentiation, proliferation, and apoptosis [5]. This has stimulated considerable scientific effort in attempts to decipher their functions in human cancers. *MiR-21* has been shown to be globally overexpressed in multiple neoplasms, including brain, lung, oesophageal, breast, thyroid, hepatocellular, pancreatic, gastric, colon, prostatic and bladder cancer, as well as in hematological malignancies [6]. Furthermore, recent reports have identified *miR-21* as a predictor of survival and therapeutic outcome in breast, colon and pancreatic cancer [7-9]. Bioinformatically predicted targets of *miR-21* that have been experimentally validated include the tumour suppressor genes: *Phosphatase and Tensin Homolog (PTEN)*, *Tropomyosin 1 (TPM1)*, *Maspin* and *Programmed Cell Death 4 (PDCD4)* [7].

PDCD4 has been shown to inhibit neoplastic transformation, tumour development and malignant progression [10,11]. In response to different inducers, *PDCD4* is upregulated in apoptosis [12]. *In vitro* and *in vivo* studies have shown that *PDCD4* suppresses the

expression of *Cyclin-Dependent Kinases 1 and 4 (CDK1, CDK4)*, *Ornithine Decarboxylase (ODC)*, *Carbonic Anhydrase Type II (CA2)*, *Jun N-Terminal Kinase (JNK)*, *Urokinase Receptor (u-PAR)*, and *Eukaryotic Translation Initiation Factor 4A (eIF4A)*, which are all responsible for promoting tumour cell proliferation, malignant progression, invasion and metastasis [13]. Very little is known however, about the upstream regulation of *PDCD4* itself. Studies have indicated that *PDCD4* expression is regulated by the *Myeloblastosis Viral Oncogene Homolog (MYB)*, *Serine/Threonine Kinase (AKT)*, *Ribosomal S6 Kinase 1 (S6K1)* and *Beta Transducin Repeat-Containing Protein (β TRCP)* [14-16]. Pharmacological manipulation using topoisomerase inhibitors and COX-2 inhibitors have been demonstrated *in vitro* to influence the expression of *PDCD4* [12,17]. Moreover, *PDCD4* expression has been shown to have significant clinical implications. Loss of *PDCD4* expression has been associated with poor patient prognosis in lung and colon cancer [18,19].

MiR-21 targets the 3'UTR of the *PDCD4* gene at nucleotides 228-249 with perfect complementarity, thereby post-transcriptionally regulating its expression [20]. Although previous studies have demonstrated that *miR-21* targets *PDCD4* and promotes cell transformation, tumour invasion and metastasis; the majority of this work has been performed on model systems including HEK-293T cells, murine JB6 cells, MCF-7, MDA-MB-231 breast cancer cell lines [21,22]. Asangani *et al* first reported that *miR-21* downregulated *PDCD4* and stimulated invasion, intravasation and metastasis in CRC cell lines and tissues [20]. However, whether this occurred at the level of gene transcription or protein translation remained unclear. The purpose of this translational investigation was to define the nature of the interaction between the *PDCD4* tumour suppressor and its oncogenic modulator *miR-21* by profiling their gene and protein expression patterns in human colorectal tissue and correlating these with patient clinicopathological data.

Materials and Methods

Colorectal tissue samples

Following ethical approval and written informed consent, primary colorectal tissues were obtained from 64 patients undergoing surgical resection or diagnostic endoscopy at Galway University Hospital, Ireland. Matched tumour-associated normal colorectal tissue was also obtained from these patients where possible. Following excision, tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Sample permitting, tissue was also immersed in formalin, prior to paraffin embedding and Consultant Histopathologist review. Prior written informed consent was obtained from each patient. Ethical approval for the study was sought and granted from the Clinical Research Ethics Committee, Galway University Hospitals, Ireland.

RNA extraction and relative quantification of miRNA and mRNA

Colorectal tissues (50 – 100mg) were homogenised using a bench-top homogeniser (Polytron® PT1600E, Kinematica AG, Littau-Luzern, Switzerland) in 1mL of QIAzol Lysis reagent (Qiagen, Crawley, UK). Total RNA was isolated from homogenised tissue using the RNeasy Tissue Mini Kit (Qiagen) as detailed in Davoren et al [23]. Concentration and purity of extracted RNA were assessed using NanoDrop 1000 spectrophotometry (NanoDrop Technologies, DE, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Electropherograms and gel-like images were evaluated using Agilent 2100 Expert software (Version B.02.03) which generated RNA integrity numbers (RIN).

Reverse-transcription quantitative PCR (RT-qPCR) was performed to quantify the expression of *miR-21* and *miR-16* (miRNA endogenous control) using sequences detailed in Chang et al [24]. Quantification of *miR-345* (miRNA endogenous control), *PDCD4*, *B2M* and *PPIA* (mRNA endogenous controls) were performed using TaqMan assays according to the manufacturer's protocols (Applied Biosystems, Foster City, CA USA). For miRNA targets 5ng of RNA was reverse-transcribed using the MultiScribe High Capacity cDNA Archive kit (Applied Biosystems). Messenger RNA was reverse transcribed using random primers (Invitrogen, USA) and SuperScript III (Invitrogen,

USA). RT-negative controls were included in each batch of reactions. RT-qPCR reactions for both miRNA and mRNA targets were carried out in final volumes of 10 μ L using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). An interassay control derived from a breast tumour specimen was included on each plate. All reactions were performed in triplicate. The threshold standard deviation for intra- and inter-assay replicates was 0.3.

MiR-16 and *miR-345* were used as endogenous controls to normalise miRNA expression levels in colorectal tissues using the $2^{-\Delta\Delta C_t}$ method [24]. *PDCD4* expression levels were normalised to *PPIA* and $\beta 2M$ expression levels [25]. Percent PCR amplification efficiencies (E) for each assay were calculated as $E = (10^{-1/\text{slope}} - 1) \times 100$, using the slope of the semi-log regression plot of C_t versus log input of cDNA (10-fold dilution series of five points). A threshold of 10% above or below 100% efficiency was applied.

PDCD4-protein Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5 μ M sections of formalin-fixed paraffin-embedded (FFPE) colorectal tissue. PDCD4 protein expression was detected was performed using rabbit polyclonal primary antibody to human PDCD4 (Abcam Inc. UK) and incubated at room temperature for 1 hour. This was followed by the addition of Ventana universal anti-mouse/anti-rabbit secondary antibody (Syntec Scientific Ltd, Ireland) for 30 minutes at room temperature. Slides were visualised using RedMap IHC Detection Kit and counterstained with hematoxylin. The intensity of PDCD4 staining was scored by two independent blinded Histopathologists as follows: Score 1 = Negative; Score 2 = Weak; Score 3 = Intermediate; Score 4 = Strong, as described [19].

Statistical analysis

Statistical analysis was performed using SPSS 14.0 (Chicago, IL, USA). Values were expressed as mean \pm s.e.m for parametric data, and median (range) for non-parametric data. Distribution of continuous data was determined using the Kolmogorov-Smirnov Z test. Differences between groups were calculated using Analysis of Variance (ANOVA) with post-hoc Tukey and Student's t-tests. Correlation between *miR-21* and *PDCD4* gene

expression was calculated using Pearson's correlation. P values of less than 0.05 were considered statistically significant for all tests.

Results

Colorectal Tissue Samples

Primary colorectal tissues consisting of 48 tumours, 61 normal tissues and 7 polyps were obtained from 64 patients. Of the 64 patients, six underwent surgery for adenomata with high grade dysplasia and one with an early stage *in situ* disease. The remaining 57 patients had histologically confirmed adenocarcinoma. All tissue samples were processed for *miR-21* and *PDCD4* gene expression. A subset of 48 parallel specimens (31 tumours and 17 normal tissues) were analysed for PDCD4 protein expression in FFPE tissue. Clinicopathological data was collected prospectively. Details of the 57 patients with cancer is summarised in Table 1. Of the 38 patients with rectal cancer, 21 had undergone neoadjuvant chemoradiotherapy. The expression of *miR-21* and *PDCD4* was confirmed to be equivalent between radiated and non-radiated tissues. Therefore all samples are included in the analysis.

Expression of miR-21 and PDCD4-mRNA

Colorectal tumours displayed an increased expression of *miR-21* compared to both normal tissues and polyps ($p = 0.005$, ANOVA). Conversely, significantly reduced expression of *PDCD4*-mRNA was observed in a progressive manner from normal, to polyp, to tumour ($p < 0.001$, ANOVA). The significance of differences between groups as determined by post-hoc analyses is shown in Figure 1. Further analysis of the reciprocal relationship between *miR-21* and *PDCD4*-mRNA expression indicated a highly significant inverse correlation ($p = 0.001$) (Figure 2).

Disease Recurrence and miR-21 Expression

After a median follow-up of 22 months (range 16-51 months), of the 51 patients who underwent curative surgery, 4 developed disease recurrence. Patients who developed recurrence displayed higher levels of *miR-21* expression than those that remained disease free (mean 2.64 and 1.87 respectively, $p < 0.001$). Interestingly, of the 15 patients who

were diagnosed with stage II disease, two developed disease recurrence. Increased expression of *miR-21* was found to be associated with recurrence in this cohort of patients (mean 2.64 and 1.78 respectively, $p = 0.122$), albeit not statistically significant due to sample size.

PDCD4-protein Immunohistochemistry

IHC was performed on 31 normal and 17 tumour specimens. Strong nuclear and cytoplasmic PDCD4 staining was observed in most normal samples (Figure 3a). In contrast, loss of nuclear staining was observed in most tumour tissues (Figure 3b). The median total PDCD4 IHC score was 5. Of 31 normal tissues, 16 had a total IHC score of greater than the median; while only 2 of 17 tumour tissues had a score of greater than 5 ($p = 0.006$). The nuclear, cytoplasmic and total PDCD4 IHC scores of these specimens are shown in Figure 4.

Discussion

Inverse Correlation Between *miR-21* and *PDCD4*-mRNA

In this the largest cohort of resected tissue specimens reported thusfar, we demonstrated that *miR-21* and *PDCD4* had reciprocal expression patterns in CRC. Prior to this, despite the perfect complementarity between *miR-21* and its target site at the 3'UTR of the *PDCD4* gene, there had been no convincing data to support the hypothesis that *miR-21* downregulates *PDCD4* by mRNA degradation. Yamamichi et al demonstrated an inverse relationship between *miR-21* and PDCD4-protein in human cell lines and resected tumour and polyp specimens [26]. The authors in this study however, did not examine PDCD4-mRNA expression. Lu et al inhibited *miR-21* expression by anti-*miR-21* in HEK-293T cells, and demonstrated a significant increase in the PDCD4 protein levels, but not at the mRNA level, suggestive that PDCD4 protein expression could be downregulated by *miR-21* through translational repression [22]. Asangani et al performed a transfection of anti-*miR-21* and pre-*miR-21* in RKO and Colo206f cell lines respectively, and observed a significant change in PDCD4 protein levels without a parallel alteration in *PDCD4*-mRNA expression [20]. This situation was not however mirrored in resected tumour specimens. In paired tumour and normal tissues from 22 colorectal cancer

patients, *miR-21* was found to be strongly inversely correlated with PDCD4 protein and weakly correlated with *PDCD4*-mRNA. The present study conducted on 49 tumour and 61 normal tissues, clearly demonstrated a strong inverse correlation between *miR-21* and *PDCD4*-mRNA expression, thus providing evidence that *miR-21* may post-transcriptionally downregulate *PDCD4* through the mechanism of mRNA degradation.

PDCD4 Protein Expression and Functions

Downregulation of *PDCD4*-mRNA resulted in a downstream reduction in protein expression as demonstrated by IHC in our study. We observed either a reduction or absence of total and nuclear PDCD4 protein expression in tumour tissue. In contrast, strong PDCD4 protein expression was observed in most normal tissues. Interestingly, cytoplasmic PDCD4 protein expression did not exhibit any distinctive pattern between tumour and normal tissues. This was not in keeping with previous reports which showed that cytoplasmic PDCD4 staining was reduced in adenomas compared with normal tissues and was absent in tumours [19]. One possible explanation for this is the ability of the PDCD4 protein to shuttle between the nucleus and cytoplasm. In one previous study, Bohm et al explored the molecular function and subcellular localisation of PDCD4 protein [27]. They demonstrated that under normal growth conditions, PDCD4 protein is located predominantly in the nucleus. However, in cells depleted of serum, most of the PDCD4 protein was found in the cytoplasm. This has been shown to be a complex subcellular import-export mechanism regulated by external signals which have yet to be identified.

It has long been established that colonic adenoma is a precursor of adenocarcinoma, and that PDCD4 is involved in cell transformation, it is therefore not surprising that PDCD4 expression in polyps were distinctly different from that of tumour or normal, and displayed a transitional phase pattern [22]. Mudduluru et al reported a large series of 71 colorectal tumour and corresponding normal tissues, together with 42 adenomata, that PDCD4 immunohistochemical staining was lost in the nucleus, and weak in the cytoplasm in tumour tissues [19]. In contrast, normal tissues exhibited strong nuclear PDCD4 staining. In the adenoma samples, there was an intermediate situation with either

intermediate nuclear or cytoplasmic staining. Our findings of intermediate PDCD4 expression in polyps were consistent with these results.

Several recent studies have elucidated some of the cellular functions of the PDCD4 protein. Talotta et al reported a novel autoregulatory pathway involving RAS, AP1 transcription factor, *miR-21* and PDCD4 [28]. The authors demonstrated that RAS induces AP1 activity, which in turn up-regulates *miR-21* expression, and leads to downregulation of PDCD4. PDCD4 subsequently acts as a negative regulator of AP1 and this completes the positive autoregulatory loop. The *miR-21/PDCD4* regulatory mechanism was shown to be critical in the RAS pathway in tumourigenesis. *PDCD4* has also recently been shown to play a role in another pathway of colorectal tumourigenesis, namely the APC, β -catenin, T-cell factor (Tcf) pathway. Wang et al initially reported that knockdown of *PDCD4* leads to downregulation of E-cadherin and activation of β -catenin/Tcf dependent transcription which promotes cell proliferation, invasion and inhibits apoptosis [29]. Subsequent study further elucidated the mechanism by which this occurs [30]. Elevated Snail expression induced by *PDCD4* knockdown activates β -catenin/Tcf transcription, which in turn stimulates the expression of c-Myc and u-PAR which are responsible for tumour invasion.

miR-21 as Prognostic Marker for Disease Recurrence

Further analysis of clinicopathological data indicated that increased *miR-21* expression was associated with disease recurrence in patients who underwent curative resection. When patients with stage II disease were analysed as a subgroup, the expression levels of *miR-21* in resected specimens was higher in those patients who subsequently developed recurrence. Analysis of additional samples will enable more definitive conclusions as clinical data accrues. Interestingly however, elevated *miR-21* expression has been established as a marker for poor prognosis in CRC [9]. Our observation that elevated *miR-21* expression was associated with early disease recurrence is not only keeping with this, but it could suggest a more refined role for *miR-21* as marker of early disease recurrence.

In summary, this study demonstrates an inverse relationship between *miR-21* and *PDCD4*. Which indicates that *miR-21* post-transcriptionally downregulates *PDCD4* by mRNA degradation through binding to its target site with perfect complementarity. This, taken together with an increasing understanding of the molecular functions of *PDCD4*, and its prognostic values, indicates that *miR-21* expression and *PDCD4* immunohistochemical staining may be of value in the diagnosis and prognostication of colorectal cancer. Manipulation of *miR-21-PDCD4* axis may represent a novel therapeutic strategy in the treatment of CRC.

Conflict of Interest Statement

The authors declare no competing interests.

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Table 1. Patient demographics and tumour characteristics (n = 57).

Characteristics	Number (percentage)
Age ¹	68.8 ± 11.9
Sex	
Male	38 (66.7)
Female	19 (33.3)
Location of tumours	
Colon	19 (33.3)
Rectum	38 (66.7)
Pathologic T classification	
Tx	5 (8.8)
T1	4 (7.0)
T2	11 (19.3)
T3	19 (33.3)
T4	16 (28.1)
Missing ³	2 (3.5)
Pathologic N classification	
Nx	3 (5.3)
N0	30 (52.6)
N1	17 (29.8)
N2	5 (8.8)
Missing ³	2 (3.5)
Metastasis classification	
M0	50 (87.7)
M1	7 (12.3)
AJCC ² classification	
Stage I	12 (21.1)
Stage II	15 (26.3)
Stage III	18 (31.6)
Stage IV	7 (12.3)
pCR	5 (8.8)

¹Mean ± standard deviation²American Joint Committee on Cancer³Two patients did not undergo surgical resection due to metastatic disease

Legends to figures**Figure 1**

Title: Differential expression of *miR-21* and *PDCD4* in colorectal tumour, polyp and normal tissues.

Legend: Progressive upregulation of (a) *miR-21* ($p = 0.004$) and downregulation of (b) *PDCD4*-mRNA ($p < 0.001$) in colorectal normal, polyp and tumour tissues. RQ = Relative Quantity calculated using the $E^{-\Delta\Delta Ct}$ method normalised to validated endogenous controls.

Figure 2

Title: Inverse correlation between *miR-21* and *PDCD4*-mRNA expression in colorectal tissues.

Legend: Expression of *PDCD4* (y-axis) plotted against *miR-21* (x-axis). Pearson correlation coefficient -0.541 , $R^2 = 0.293$, $p < 0.001$. RQ = Relative Quantity calculated using the $E^{-\Delta\Delta Ct}$ method normalised to validated endogenous controls.

Figure 3

Title: *PDCD4* immunochemical staining of normal ($n = 31$) and tumour ($n = 17$) tissues.

Legend: (a) Normal tissue displayed strong nuclear and cytoplasmic *PDCD4* staining. (b) Loss of nuclear staining and weak cytoplasmic staining was observed in tumour tissue. The median and range of the intensity score of all tumour and normal tissues are shown.

Figure 4

Title: Total, nuclear and cytoplasmic PDCD4 IHC scores of normal (n = 31) and tumour tissues (n = 17).

Legend: (a, b) The majority of normal tissues displayed intermediate or strong total and nuclear staining. In contrast, most tumour tissues had a low total PDCD4 IHC score and loss of nuclear staining was observed. (c) Overall cytoplasmic IHC score did not exhibit distinctive pattern between tumour and normal tissues.

