<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Clinical Applications of Molecular Profiling in Colorectal Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Kheirelseid, Elrasheid Ahmed Hassan</td>
</tr>
<tr>
<td><strong>Publication Date</strong></td>
<td>2011-06</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/2961">http://hdl.handle.net/10379/2961</a></td>
</tr>
</tbody>
</table>

Some rights reserved. For more information, please see the item record link above.
MicroRNA-21 and PDCD4 expression in colorectal cancer

K.H. Chang a, N. Miller a,*, E.A.H. Kheirelseid a, H. Ingoldsby b, E. Hennessy a, C.E. Curran a, S. Curran b, M.J. Smith a, M. Regan a, O.J. McAnena a, M.J. Kerina

a Department of Surgery, National University of Ireland, Galway, Ireland
b Department of Pathology, National University of Ireland, Galway, Ireland

Accepted 11 April 2011
Available online 4 May 2011

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 tissues. 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.

© 2011 Elsevier Ltd. All rights reserved.

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. 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. 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. 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. MiRNAs play major roles in governing diverse biological processes such as differentiation, proliferation, and apoptosis. 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 haematological
malignancies. Furthermore, recent reports have identified miR-21 as a predictor of survival and therapeutic outcome in breast, colon and pancreatic cancer.\textsuperscript{7,8,9} 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).\textsuperscript{7} PDCD4 has been shown to inhibit neoplastic transformation, tumour development and malignant progression.\textsuperscript{10,11} In response to different inducers, PDCD4 is upregulated in apoptosis.\textsuperscript{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.\textsuperscript{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 (βTRCP).\textsuperscript{14–16} Pharmacological manipulation using topoisomerase inhibitors and COX-2 inhibitors have been demonstrated in vitro to influence the expression of PDCD4.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{21,22} Asangani et al first reported that miR-21 downregulated PDCD4 and stimulated invasion, intravasation and metastasis in CRC cell lines and tissues.\textsuperscript{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 (50–100 mg) were homogenised using a bench-top homogeniser (Polytron® PT1600E, Kinematica AG, Littau-Lazem, Switzerland) in 1 mL 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.\textsuperscript{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.\textsuperscript{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 5 ng 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 μL using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). An inter-assay 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−DDCt method.\textsuperscript{24} PDCD4 expression levels were normalised to PPIA and β2M expression levels.\textsuperscript{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 Ct 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 μM sections of formalin-fixed paraffin-embedded (FFPE) colorectal tissue. PDCD4 protein expression was performed using rabbit polyclonal primary antibody to human PDCD4 (Abcam Inc. UK) and incubated at room temperature for 1 h. This was followed by the addition of Ventana universal anti-mouse/anti-rabbit secondary antibody (Syntec Scientific Ltd, Ireland) for 30 min at room temperature. Slides were visualised using RedMap IHC Detection Kit and counterstained with haematoxylin. 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 ± 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-mRNA expression was calculated using Pearson’s correlation. \( p \text{ values of less than 0.05 were considered statistically significant for all tests.} \)

**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 \( \text{in situ} \) disease. The remaining 57 patients had histologically confirmed adenocarcinoma. All tissue samples were processed for \( \text{miR}-21 \) and \( \text{PDCD4} \) 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 \( \text{miR}-21 \) and \( \text{PDCD4} \) was confirmed to be equivalent between radiated and non-radiated tissues. Therefore all samples are included in the analysis.

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

\(^a\) Mean ± standard deviation.  
\(^b\) American Joint Committee on Cancer.  
\(^c\) Two patients did not undergo surgical resection due to metastatic disease.

**Expression of miR-21 and PDCD4-mRNA**

Colorectal tumours displayed an increased expression of miR-21 compared to both normal tissues and polyps \( (p = 0.005, \text{ANOVA}) \). Conversely, significantly reduced expression of PDCD4-mRNA was observed in a progressive manner from normal, to polyp, to tumour \( (p < 0.001, \text{ANOVA}) \). The significance of differences between groups as determined by post-hoc analyses is shown in Fig. 1. Further analysis of the reciprocal relationship between \( \text{miR}-21 \) and PDCD4-mRNA expression indicated a highly significant inverse correlation \( (p = 0.001) \) (Fig. 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 \( \text{miR}-21 \) 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 \( \text{miR}-21 \) 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 (Fig. 3a). In contrast, loss of nuclear staining was observed in most tumour tissues (Fig. 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 Fig. 4.

**Discussion**

**Inverse correlation between miR-21 and PDCD4-mRNA**

In this the largest cohort of resected tissue specimens reported thus far, 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

---

**Figure 1.** Differential expression of miR-21 and PDCD4 in colorectal tumour, polyp and normal tissues. 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\(^{-\Delta\Delta C_{T}}\) method normalised to validated endogenous controls.

**Figure 2.** Inverse correlation between miR-21 and PDCD4-mRNA expression in colorectal tissues. Expression of PDCD4 \( (y\text{-axis}) \) plotted against miR-21 \( (x\text{-axis}) \). Pearson correlation coefficient \(-0.541, R^2 = 0.293, p < 0.001.\) RQ = Relative Quantity calculated using the E\(^{-\Delta\Delta C_{T}}\) method normalised to validated endogenous controls.
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. 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. 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. Mudduluru et al reported a large series of 71 colorectal tumour and corresponding normal tissues, together with 42 adenomata, that PDCD4 immunohistochemical

---

**Figure 3.** PDCD4 immunochemical staining of normal (n = 31) and tumour (n = 17) tissues. (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.** Total, nuclear and cytoplasmic PDCD4 IHC scores of normal (n = 31) and tumour tissues (n = 17). (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.
staining was lost in the nucleus, and weak in the cytoplasm in tumour tissues. 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. 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. Subsequent study further elucidated the mechanism by which this occurs. 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 is 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. 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 miRNA 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.

**Acknowledgements**

This work was funded by the National Breast Cancer Research Institute, Galway, Ireland. The authors gratefully acknowledge Dr. John Newell, Health Research Board Clinical Research Facility, National University of Ireland, Galway for his continuing biostatistical input.

**Conflict of interest**

The authors declare no competing interests.

**References**


