MicroRNA-21 promotes tumour malignancy via increased nuclear translocation of β-catenin and predicts poor outcome in APC-mutated but not in APC-wild-type colorectal cancer

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MicroRNA-21 (MiR-21) overexpression is commonly observed in colon adenocarcinomas. However, in our preliminary data, the prognostic value of miR-21 levels was observed only in adenomatous polyposis coli (APC)-mutated tumours, not in APC-wild-type tumours. We explored whether β-catenin nuclear translocation was synergistically promoted by miR-21 in APC-mutated cells but not in APC-wild-type cells. We enrolled 165 colorectal tumour to determine APC mutation, miR-21 levels and nuclear β-catenin expression by direct sequencing, real-time PCR and immunohistochemistry. Overall survival and relapse-free survival were analysed by Kaplan–Meier and Cox regression models. The mechanistic action of β-catenin nuclear translocation modulated by miR-21 and its effect on cell invasion were evaluated in a cell model. Positive nuclear β-catenin expression was more commonly occurred in APC-mutated tumours than in APC-wild-type tumours. High miR-21 levels were relatively more common in tumours with positive nuclear β-catenin expression than in those with negative nuclear β-catenin expression. APC-mutated tumours with high miR-21 levels had shorter overall survival and relapse-free survival periods compared with others. However, the prognostic value of miR-21 levels was not observed in APC-wild-type tumours. Phosphorylation of β-catenin at Ser552 by AKT plays a critical role in β-catenin nuclear translocation in APC-mutated but not in APC-wild-type cells. Moreover, nuclear β-catenin expression increased by AKT is responsible for the capability of invasiveness. In summary, nuclear translocation of β-catenin increased by miR-21 promotes tumour malignancy and a poor outcome in APC-mutated patients but not in APC-wild-type colorectal cancer.

Introduction

Colorectal cancer has the highest incidence and is the third leading cause of cancer death in Taiwan; however, the prognosis of patients with colorectal cancer is relatively more favourable when compared with other cancers (1). Therefore, establishing reliable prognostic and therapeutic markers is useful to improve the outcome in colorectal cancer patients. The majority of colorectal tumours have a mutation in a key regulatory factor of the Wnt/β-catenin pathway, resulting in activation of the pathway. The mutations were most often in adenomatous polyposis coli (APC) and rarely occurred in β-catenin (2–6). Up to 80% of tumours have a nuclear accumulation of β-catenin to cooperate with T-cell factor (TCF) and in turn up-regulate the expression of downstream genes of the Wnt/β-catenin pathway, such as c-Myc and cyclin D1 (7–10). Activation of downstream genes of the Wnt/β-catenin pathway by APC mutation was shown to be linked with the initiation of colorectal tumourigenesis (11,12). However, the prognostic significance of APC mutation in colorectal cancer has not yet been reported. This observation suggests that the APC mutation could not be fully responsible for β-catenin nuclear translocation through the activation of the Wnt/β-catenin pathway and consequently for the promotion of tumour malignancy and the poor outcome in colorectal cancer.

Materials and methods

Study subjects

This study included 165 resected tumours from colorectal cancer patients recruited from the Colorectal Division, Department of Surgery, Chung Shan Medical University Hospital, Taichung, Taiwan, between 2000 and 2007. All subjects were unrelated ethnic Chinese residents of central Taiwan and provided written informed consent approved by the Institutional Review Board. Among these patients, 30 had tumour metastases in the liver (12 cases), omentum (5 cases), lung (3 three cases), hypopharynx, bone, left para-aortic lymph node, pelvis, rectum and mesentery (all one case); the metastatic data of five patients were not available. In total, three patients had tumours that metastasized to more than one organ. No patient received chemotherapy or radiotherapy before surgical resection. The overall survival (OS) of patients was calculated from the date of surgery. Board-certified pathologists conducted examinations for the pathological stage of each case. Information pertaining to personal characteristics was collected from hospital reports.

Real-time PCR analysis of miR-21 and cyclin D1 mRNA expression levels

DNase I-treated total RNA was subjected to miRNA real-time PCR analysis with the TaqMan miRNA Reverse Transcription Kit, miRNA assays and an Real-Time Thermocycler 7500 (Applied Biosystems, Foster City, CA). RNU6B was used as the small RNA reference housekeeping gene. The primers used for real-time PCR analysis of cyclin D1 mRNA expression are shown in Supplementary Table 1, available at Carcinogenesis Online. The miR-21 levels in tumours that were higher than the median value were defined as ‘high’, whereas levels lower than the median value were defined as ‘low’.

Immunohistochemical staining

The β-catenin expression in paraffin sections of colorectal tumours from primary colorectal cancer patients was evaluated by immunohistochemistry
using β-catenin antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and immunohistochemical procedures described previously (18). Samples did not treat with the primary antibody served as negative controls. The immunohistochemical staining scores were defined as described previously (19,20), and the signal intensities were evaluated independently by three observers. Immunostaining scores were defined as the cell staining intensity (0 = nil; 1 = weak; 2 = moderate and 3 = strong) multiplied by the percentage of labelled cells (0–100%), leading to scores from 0 to 300. A score >150 was defined as ‘high’ immunostaining, whereas a score <150 was defined as ‘low’.

APC sequencing
APC mutations in colorectal cancer patients were detected in the mutation cluster region. The mutations were determined by direct sequencing of PCR products amplified from the DNA of tumour cells isolated by the colorectal tumour tissues. These primers are shown in Supplementary Table 1, available at Carcinogenesis Online. The PCR products were sequenced using an Applied Biosystems 3100 Avant Genetic Analyser (Applied Biosystems), and the same primers were used for both the PCR and the DNA sequencing. All mutations were confirmed by direct sequencing of both DNA strands.

Cell lines
LoVo, HCT15 and HT29 cells were kindly provided by Dr S.-G.Shia (National Institute of Cancer Research, National Health Research Institutes, Miaoli, Taiwan). HCT116 cells were kindly provided by Dr C.C.Chang (Institute of Biomedical Sciences, National Chung Hsing University, Taichung, Taiwan). SW480 cells were obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). HCT15, HCT116 and HT29 colon cancer cell lines were maintained in RPMI-1640 (HyClone, Logan, UT), SW480 colon cancer cell lines were maintained in L-15 (HyClone), and LoVo colorectal cancer cells were maintained in F12-K (HyClone). The medium contained 10% fetal bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). These cells were cultured according to the suppliers’ instructions and were stored used at passages 5–20. Once resuscitated, cell lines were routinely authenticated (once every 6 months; cells were last tested in December 2012) through cell morphology monitoring, growth curve analysis, species verification by isoenzymology and karyotyping, identity verification using short tandem repeat profiling analysis and contamination checks.

Plasmid construction, transfection and stable clone selection
β-catenin and APC shRNA were purchased from the National RNAi Core Facility, Academia Sinica, Taiwan, and the sequences were shown in Supplementary Table 1, available at Carcinogenesis Online. The seed sequences of two β-catenin shRNA were located at 3′UTR of β-catenin (Supplementary Figure 1A, available at Carcinogenesis Online). However, the plasmid-derived shRNA (miR-21) did not contain 3′UTR. Two stable clones of β-catenin-knockdown HCT15 and HCT116 cells were selected by puromycin. The expression vector of β-catenin and APC were purchased from Addgene (Cambridge, MA). Non-specific shRNA control of the scramble sequence was used as the control in the knockdown experiment, and a vector control was used as the control of β-catenin overexpression. β-catenin-ssS52A was determined as the scrambled nucleotide-directed mutation system (Stratagene, Santa Clara, CA); the primers are shown in Supplementary Table 1, available at Carcinogenesis Online. The transfection and stable clone selection procedures were as described previously (19).

miR-21 precursor and inhibitor transfection
Six-well plates were used to confluence the cells. MiR-21 precursor (pre-miR-21, 20–40 nmol/l per well; Ambion, Foster City, CA) or miR-21 inhibitor (40–80 nmol/l per well; Ambion) and negative control cells (Ambion) were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Foster City, CA) according to the manufacturers’ protocols. Transfection efficiency was evaluated by real-time PCR.

Luciferase reporter assay
Cells were transfected with TOPFLASH and FOPFLASH (Millipore, Billerica, MA). Luciferase assays were performed using the Luciferase Reporter Assay System (Promega, Madison, WI) 24 h after transfection. Normalized luciferase activity was reported as the ratio of luciferase activity/β-galactosidase activity.

Boyden chamber invasion assay
A Boyden chamber with a pore size of 8 μm was used for the in vitro cell invasion assay. Cells (5×10⁴) in 0.5% serum containing the culture medium were plated in the upper chamber, and 10% fetal bovine serum was added to the culture medium in the lower chamber as a chemoattractant. The upper side of the filter was covered with 0.2% Matrigel (Collaborative Research, San Francisco, CA) diluted in RPMI-1640. After 24 h, cells on the upper side of the filter were removed, and cells that adhered to the underside of the membrane were fixed in 95% ethanol and stained with 10% Giemsa dye. The number of invasive cells was counted. Ten contiguous fields of each sample were examined to obtain a representative number of cells that invaded across the membrane. The procedures and methods were as previously described (21).

Statistical analysis
All statistical analyses were performed using the SPSS statistical software program as described previously (version 11.0; SPSS, Chicago, IL) (19,22). Statistical tests were examined by two-sided analysis of the variance, and P values <0.050 were considered statistically significant.

Results
Nuclear β-catenin expression correlated with APC mutations and miR-21 expression in tumours from colorectal cancer patients
The expression levels of miR-21 and APC mutations in 165 colorectal tumours were examined by real-time PCR and direct sequencing to evaluate whether both molecules could be associated with patients’ clinical parameters. As shown in Supplementary Tables 2 and 3, available at Carcinogenesis Online, miR-21 expression levels and APC mutation were not associated with clinical parameters, including age, gender, tumour site, cigarette smoking and tumour stage.

β-catenin was evaluated by immunohistochemistry and the representative immunostaining results are shown in Supplementary Figure 2, available at Carcinogenesis Online. There was a higher prevalence of positive nuclear β-catenin expression in APC-mutated tumours than in APC-wild-type tumours (P = 0.003; Table I). Interestingly, high miR-21 levels were relatively more common in tumours with positive nuclear β-catenin, but this did not reach statistical significance (P = 0.182; Table I). When tumours were divided into four subgroups by two parameters of APC mutation and miR-21, the highest prevalence of positive nuclear β-catenin expression was observed in APC-mutated tumours with high miR-21 levels compared with the other three subgroups (85.7% versus 45.6, 25.0 and 28.0%, P <0.001; Table I). These results suggest that miR-21 might promote nuclear translocation of β-catenin in patients with APC-mutated tumours.

MiR-21 expression levels are associated with poor OS and relapse-free survival in APC-mutated patients but not in APC-wild-type patients
As previously mentioned, miR-21 expression levels have been associated with poor prognosis in colon adenocarcinoma patients (13,15). Herein, the usefulness of miR-21 expression levels was evaluated for predicting the outcome in colorectal cancer. Kaplan–Meier analysis showed that miR-21 and APC mutations were not associated with OS and relapse-free survival (RFS) in patients studied (data not shown). When tumours were divided into four subgroups by both parameters (P = 0.045 for OS; P = 0.019 for RFS; Figure 1A and B), APC-mutated tumours with high miR-21 levels had shorter OS and RFS periods than APC-wild-type tumours with high miR-21 levels (P = 0.035 for OS; P = 0.024 for RFS; Figure 1A and B) or APC-mutated tumours with high miR-21 levels (P = 0.031 for OS; P = 0.044 for RFS; Figure 1A and B). The prognostic significance was not observed between APC-mutated patients with high miR-21 levels versus APC-wild-type patients with low miR-21 levels (P = 0.392 for OS; P = 0.412 for RFS; Figure 1A and B) and APC-mutated patients with low miR-21 levels versus APC-wild-type patients with high miR-21 levels (P = 0.547 for OS; P = 0.701 for RFS; Figure 1A and B). In addition, poorer OS and RFS in APC-mutated patients with high miR-21 levels was still observed compared with the combination of the other three subgroups (P = 0.019 for OS, Figure 1C; P = 0.014 for RFS, Figure 1D). Cox regression analysis indicated that APC-mutated patients with high miR-21 levels had a hazard ratio (HR) of 1.98 and 1.59 for OS and RFS, respectively, compared with APC-mutated patients with low miR-21 tumours (95% confidence interval [CI]: 1.14–3.24, P = 0.045 for OS; 95% CI: 1.04–3.56, P = 0.047 for RFS; Table II). However, the prognostic value of miR-21 levels for OS and RFS was not observed in APC-wild-type patients (Table II). Interestingly, APC-mutated patients with high miR-21 levels had poorer OS and RFS than did APC-wild-type patients with...
MiR-21 enhances β-catenin nuclear translocation via increased phosphorylation of β-catenin at Ser552

Phosphorylation of β-catenin at Ser552 is responsible for β-catenin nuclear translocation and its transcriptional activity in APC-mutated but not in APC-wild-type colon cancer cells

To confirm the efficacy of β-catenin silenced by shRNA, two β-catenin shRNA were used to knock down β-catenin in HCT15 and HCT116 cells. TCF-reporter activity assay showed that a similar reduction of TCF-reporter activity was observed in both cells transfected with two β-catenin shRNA (Supplementary Figure 1B, available at Carcinogenesis Online). To exclude the clonal effects, two stable clones of β-catenin knockdown of HCT15 and HCT116 were established and a similar reduction of TCF-reporter activity was seen in two stable clones of both cells (Supplementary Figure 1C, available at Carcinogenesis Online). We next explored whether phosphorylation of β-catenin at Ser552 could be responsible for its nuclear translocation and transcriptional activity in APC-mutated but not APC-wild-type colon cancer cells. To test the hypothesis, one of β-catenin-knockdown (shβ2-catenin) HCT15 stable clone with mutated APC was used to introduce the expression vector of wild-type β-catenin or β-catenin with the S552A mutation (β-catenin-S552A) and/or treatment of the miR-21 precursor. Western blotting showed that nuclear β-catenin expression increased markedly in HCT15 cells with wild-type β-catenin transfection plus miR-21 precursor treatment, but nuclear β-catenin expression was slightly elevated by wild-type β-catenin alone (Figure 4A, upper panel). However, nuclear β-catenin expression was not detected in HCT15 cells transfected with β-catenin-S552A transfection and/or combined with the miR-21 precursor (Figure 4A, upper panel). Consistently, cytoplasmic β-catenin disappeared in HCT15 cells with wild-type β-catenin transfection plus the miR-21 precursor (Figure 4A, upper panel). Cytoplasmic β-catenin expression was observed in HCT15 cells with β-catenin-S552A transfection and/or combined with the miR-21 precursor (Figure 4A, upper panel). A marked increase in TCF-reporter activity and invasion capability were observed in HCT15 cells with wild-type β-catenin plus the miR-21 precursor compared with other treatments (Figure 4A, lower panel, and Figure 4B). Nuclear β-catenin expression level, TCF-reporter activity and invasion capability were not elevated by the miR-21 precursor or miR-21 inhibitor in the shβ2-catenin HCT116 stable clone with wild-type β-catenin transfection, when compared with the shβ2-catenin HCT116 stable clone without wild-type β-catenin transfection and/or the miR-21 precursor or inhibitor co-treatment (Figure 4B). In addition, shβ2-catenin HCT116 stable clones with wild-type β-catenin transfection were co-transfected with APC shRNA (shAPC) and/or miR-21 precursor

Table I. Relationships between β-catenin protein nuclear expression and clinical-pathological parameters in colorectal cancer patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Case no.</th>
<th>Nucleus β-catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Age ≤65</td>
<td>90 (54.5)</td>
<td>75 (45.5)</td>
</tr>
<tr>
<td>Age &gt;65</td>
<td>36 (47.4)</td>
<td>40 (52.6)</td>
</tr>
<tr>
<td>Gender Female</td>
<td>40 (52.6)</td>
<td>36 (47.4)</td>
</tr>
<tr>
<td>Gender Male</td>
<td>50 (56.2)</td>
<td>39 (43.8)</td>
</tr>
<tr>
<td>Tumour site Proximal colon</td>
<td>21 (52.5)</td>
<td>19 (47.5)</td>
</tr>
<tr>
<td>Tumour site Distal colon</td>
<td>30 (55.6)</td>
<td>24 (44.4)</td>
</tr>
<tr>
<td>Tumour site Rectum</td>
<td>39 (54.9)</td>
<td>32 (45.1)</td>
</tr>
<tr>
<td>Smoking status Non-smoking</td>
<td>58 (53.2)</td>
<td>51 (46.8)</td>
</tr>
<tr>
<td>Smoking</td>
<td>32 (57.1)</td>
<td>24 (42.9)</td>
</tr>
<tr>
<td>Stage I + II</td>
<td>39 (56.5)</td>
<td>30 (43.5)</td>
</tr>
<tr>
<td>Stage III</td>
<td>35 (53.0)</td>
<td>31 (47.0)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>16 (53.3)</td>
<td>14 (46.7)</td>
</tr>
<tr>
<td>APC mutation No</td>
<td>67 (63.8)</td>
<td>38 (36.2)</td>
</tr>
<tr>
<td>APC mutation Yes</td>
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<td>37 (61.7)</td>
</tr>
<tr>
<td>miR-21 Low</td>
<td>49 (59.8)</td>
<td>33 (40.2)</td>
</tr>
<tr>
<td>miR-21 High</td>
<td>41 (49.4)</td>
<td>42 (50.6)</td>
</tr>
<tr>
<td>APCntmut/miR-21 status No</td>
<td>31 (54.4)</td>
<td>26 (45.6)</td>
</tr>
<tr>
<td>APCntmut/miR-21 status Yes</td>
<td>18 (72.0)</td>
<td>7  (28.0)</td>
</tr>
<tr>
<td>APCmut/miR-21 status Yes</td>
<td>5 (14.3)</td>
<td>30 (85.7)</td>
</tr>
</tbody>
</table>

Table II. Expression of APC and miR-21 in five types of colon cancer cells

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Case no.</th>
<th>Nucleus β-catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (%)</td>
</tr>
</tbody>
</table>
| high miR-21 levels (HR: 2.73, 95% CI: 1.37–5.46, P = 0.004 for OS; HR: 2.88, 95% CI: 1.46–5.57, P = 0.02 for RFS; Table II). These results suggest that miR-21 levels may be useful to predict the outcome in APC-mutated patients but not in APC-wild-type patients.

Cell invasion capability is dependent on miR-21 expression in colon cancer cells with APC or β-catenin mutation

Five types of colon cancer cells (HCT15, SW480, HT29, LoVo and HCT116) were used to test whether miR-21 expression could modulate cell invasion capability; HCT15, SW480, HT29 and LoVo possessed APC mutations, and HCT116 harboured β-catenin codon 45 mutations. As shown in Figure 2A, the highest miR-21 expression level was found in HCT116 cells, followed by LoVo, HT29, SW480 and HCT15 cells. MiR-21 expression was knocked down by miR-21 inhibitor in HCT116 and LoVo cells and ectopically expressed by miR-21 precursor in HCT15 and SW480 cells (Figure 2B). As expected, miR-21 expression levels decreased in miR-21 knockdown HCT116 and LoVo cells and increased in miR-21 overexpressing HCT15 and SW480 cells. Boyden chamber and MTT assays showed that the invasion and cell growth capability decreased markedly in miR-21 knockdown HCT116 and LoVo cells and increased in miR-21 overexpressing HCT15 and SW480 cells in a dose-dependent manner (Figure 2C). Representative invasive cells on Matrigel membranes are shown in Figure 2C (right panel). However, cell proliferation was not affected by miR-21 precursor and miR-21 inhibitor in these cells (Supplementary Figure 3, available at Carcinogenesis Online). These results clearly indicate that miR-21 promotes invasiveness but does not affect cell proliferation in colon cancer cells.

**MiR-21 promotes β-catenin nuclear translocation via increased phosphorylation of β-catenin at Ser552**

Phosphorylation of β-catenin at Ser552 by AKT promotes β-catenin transcriptional activity and, in turn, enhances tumour invasion (17).
Table II. Cox regression analysis for the combined effects of APC mutation and miR-21 on OS and RFS in colorectal cancer patients

<table>
<thead>
<tr>
<th>APC mutation /miR-21</th>
<th>OS</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case no.</td>
<td>5-year survival (%)</td>
</tr>
<tr>
<td>No/high</td>
<td>41</td>
<td>66</td>
</tr>
<tr>
<td>Yes/high</td>
<td>35</td>
<td>42</td>
</tr>
<tr>
<td>Yes/low</td>
<td>25</td>
<td>64</td>
</tr>
<tr>
<td>No/low</td>
<td>48</td>
<td>39</td>
</tr>
<tr>
<td>Yes/low</td>
<td>25</td>
<td>64</td>
</tr>
<tr>
<td>No/low</td>
<td>48</td>
<td>39</td>
</tr>
<tr>
<td>No/high</td>
<td>41</td>
<td>66</td>
</tr>
<tr>
<td>Yes/high</td>
<td>35</td>
<td>42</td>
</tr>
</tbody>
</table>

HR: adjusted by age, genders, smoking status and stage.
to examine whether miR-21 could enhance β-catenin nuclear translocation, TCF-reporter activity and invasion capability. As shown in Figure 4C, the levels of phosphorylated β-catenin at Ser552 in shβ-catenin HCT116 stable clone were markedly increased by wild-type β-catenin plus shAPC transfection compared with shβ-catenin HCT116 stable clone with wild-type β-catenin transfection. The level of phosphorylated β-catenin at Ser552 was more elevated by the miR-21 precursor in the shβ-catenin HCT116 stable clone with wild-type β-catenin plus shAPC transfection. Interestingly, the TCF-reporter activity and invasion capability was dependent on the levels of phosphorylated β-catenin at Ser552 in the shβ-catenin HCT116 stable clone with wild-type β-catenin plus shAPC transfection and/or miR-21 precursor. On the other hand, no influence of the miR-21 precursor or miR-21 inhibitor on β-catenin nuclear translocation was observed in the shAPC HCT15 stable clone with wild-type APC transfection even though the phosphorylated β-catenin expression level was elevated by miR-21 precursor (Supplementary Figure 4, available at Carcinogenesis Online). Collectively, these results indicate that miR-21 promotes β-catenin nuclear translocation, TCF-reporter activity and invasion capability in APC-mutated (or silencing) but not in APC-wild-type colon cancer cells.

Discussion

MiR-21 has been associated with poor prognosis via the promotion of colorectal tumour progression and metastasis. However, the role of miR-21 in colorectal tumour malignancy remains unclear. In the present study, an increase in β-catenin nuclear translocation by miR-21 was responsible for miR-21-mediated invasiveness in APC-mutated cells but not in APC-wild-type colon cancer cells. Among patients, nuclear β-catenin expression positively correlated with APC mutation and miR-21 levels in colorectal tumours. The prognostic value of miR-21 levels on OS and RFS was observed only in APC-mutated patients but not in APC-wild-type patients. In addition, consistent with a previous study (23), the correlation of miR-21 expression with APC mutation and nuclear β-catenin expression was observed in advanced patients of this study population (Supplementary Table 4, available at Carcinogenesis Online). Therefore, we suggest that miR-21 levels may serve to predict the outcome in APC-mutated patients but not in APC-wild-type patients.

APC functions in a normal colon to negatively regulate Wnt signalling through targeting of β-catenin for proteasome degradation (24). Wild-type APC plays a central role in a destruction complex that includes Axin, glycogen synthase kinase-3β (GSK-3β) and casein kinase 1 (CK1) (25,26). This complex directs a series of phosphorylation events on β-catenin that targets it for ubiquitination and subsequent proteolysis (25). The canonical mechanism of β-catenin regulation involves a destruction complex where β-catenin is phosphorylated by GSK-3β at the Thr41, Ser45 and Ser37 sites (27,28). Our data showed that the expression levels of phosphorylation of β-catenin at Ser37 and Thr41/Ser45 did not change by miR-21 overexpression in HCT15 and HCT116 cells (Supplementary Figure 5, available at Carcinogenesis Online), suggesting that GSK-3β might play a minor role in miR-21-mediated β-catenin nuclear translocation.

A recent report using zebrafish and human cells showed that homozygous loss of APC causes failed intestinal cell differentiation due to the elevation of C-terminal-binding protein-1 (CtBP1) (6). Increased nuclear β-catenin and intestinal proliferation require the additional activation of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (6). Therefore, expression of CtBP1 elevated by APC loss contributes to adenoma initiation, whereas KRAS activation and β-catenin nuclear localization promote adenoma progression to carcinoma (6). Therefore, APC loss alone results in cytoplasmic β-catenin accumulation but does not enhance β-catenin nuclear translocation. Phosphorylation of β-catenin at Ser552 by AKT plays an important role in increased nuclear translocation and transcriptional activity of the β-catenin/TCF axis (17). Consistently, the expression levels of phosphorylated β-catenin at Ser552 in HCT15 and HCT116
Fig. 3. MiR-21 promotes β-catenin/TCF transcriptional activity in colon cancer cells. A precursor and inhibitor of miR-21 were respectively treated with (A) HCT15 and HCT116 for 48 h, and then western blotting was used to determine β-catenin expression in whole-cell extracts and cytoplasmic and nuclear protein fractions. β-actin, α-tubulin and Sp1 were used as protein-loading controls of whole-cell extracts and cytoplasmic and nuclear fractions, respectively. (B) TOPFLASH and FOPFLASH were, respectively, co-transfected with miR-21 precursor and inhibitor into HCT15 and HCT116 for 48 h. Reporter activity was determined using luciferase reporter assay. β-gal served as an internal control. The changes of cyclin D1 mRNA expression levels by the miR-21 precursor and inhibitor were evaluated by real-time PCR.
MiR-21 enhances β-catenin nuclear translocation. However, the combination of β-catenin S552A and miR-21 precursor was transduced into the HCT15 shβ-catenin stable clone, and western blotting was used to evaluate β-catenin expression in whole-cell extracts and cytoplasmic and nuclear protein. TOPFLASH was respectively transfected into those groups for 24 h, and then the reporter activity was determined using the luciferase reporter assay. β-gal served as an internal control. The Boyden chamber invasion assay was performed to evaluate changes in invasiveness. The invasive cells on Matrigel membranes are shown as representative pictures in the upper panel and a quantitative graph in the lower panel.

TOPFLASH was co-transduced into the shβ-catenin HCT116 stable clone with different treatments for 24 h, and then reporter activity was determined by a luciferase reporter activity assay. β-gal served as an internal control (middle panel). The Boyden chamber invasion assay was performed to evaluate the changes of invasiveness. Representative invasive cells on Matrigel membranes and a quantitative graph are shown in the lower panel.

Wild-type β-catenin, miR-21 precursor and miR-21 inhibitor were transfected into the shβ-catenin HCT116 stable clone, and western blotting was used to evaluate β-catenin expression in whole-cell extracts and the cytoplasmic and nuclear fraction of cells (upper panel). TCF-reporter activity (TOPFLASH transfection for 24 h) was evaluated by the luciferase reporter activity assay in the shβ-catenin HCT116 stable clone with different treatments (middle panel). β-gal served as an internal control. The Boyden chamber invasion assay was performed to evaluate the invasiveness of the shβ-catenin HCT116 stable clone with different treatments. Representative invasive cells on Matrigel membranes are shown in the lower panel, and a quantitative graph is shown in the upper panel.

Phosphorylation of β-catenin at Ser552 is responsible for β-catenin nuclear translocation in colon cancer cells with mutant APC or with mutant β-catenin. (A) Wild-type β-catenin, β-catenin S552A and/or miR-21 precursor were transfected into the HCT15 shβ-catenin stable clone, and western blotting was used to evaluate β-catenin expression in whole-cell extracts and cytoplasmic and nuclear protein. TOPFLASH was respectively transfected into those groups for 24 h, and then the reporter activity was determined using the luciferase reporter assay. β-gal served as an internal control. The Boyden chamber invasion assay was performed to evaluate changes of invasiveness. The invasive cells on Matrigel membranes are shown as representative pictures in the upper panel and a quantitative graph in the lower panel. (B) Wild-type β-catenin, miR-21 precursor and miR-21 inhibitor were transfected into the shβ-catenin HCT116 stable clone, and western blotting was used to evaluate β-catenin expression in whole-cell extracts and the cytoplasmic and nuclear fraction of cells (upper panel). TCF-reporter activity (TOPFLASH transfection for 24 h) was evaluated by the luciferase reporter activity assay in the shβ-catenin HCT116 stable clone with different treatments (middle panel). β-gal served as an internal control. The Boyden chamber invasion assay was performed to evaluate the invasiveness of the shβ-catenin HCT116 stable clone with different treatments. Representative invasive cells on Matrigel membranes are shown in the lower panel, and a quantitative graph is shown in the upper panel.

Cells increased by miR-21 overexpression in a dose-dependent manner (Figure 3A). This may partially explain why the elevation of nuclear β-catenin expression was observed in miR-21 overexpressing HCT15 and HCT116 cells even though both cells harboured KRAS mutations (29).

PTEN is targeted by miR-21 and, in turn, activates the PI3K/AKT signalling pathway (30). β-Catenin activation synergizes with PTEN loss to promote tumour progression and metastasis in melanomas, bladder, endometrial and prostate cancers (31–34). Ya et al. showed that miR-21 induced colon cancer cell stemness via activation of the Wnt/β-catenin signalling pathway by down-regulating transforming growth factor beta, receptor 2 (TGF-βR2) (35). Recently, an animal model study showed that TGF-βR2 knockout alone was not sufficient for intestinal tumour formation, and lack of PTEN alone had a weak effect on intestinal tumour induction (36). However, the combination of TGF-βR2 inactivation with PTEN loss led to malignant tumours in both the small intestine and the colon in 86% of the mice and to metastasis in 8% of the tumour-bearing mice (36). Therefore, inactivation of the TGF-β signalling pathway combined with loss of PTEN cooperatively promotes intestinal tumour progression (36). In the present study, we provided evidence that miR-21 promoted the invasive capability of APC-mutated colon cancer cells via increased β-catenin nuclear localization and transcriptional activity of the β-catenin/TCP signalling pathway. We also observed that the TCF-reporter activity enhanced by miR-21 overexpression in HCT15 cells was restored by NF-κB inhibitor BAY and PI3K/AKT inhibitor LY94002 but not by ERK inhibitor AZD6244. Similarly, the TCF-reporter activity promoted by miR-21 overexpression in HCT116 cells decreased by BAY and LY294002 but not by AZD6244 (Supplementary Figure 6, available at Carcinogenesis Online). These results are consistent with previous studies showing that miR-21 expression is regulated by the NF-κB signalling pathway (37). Phosphorylation of β-catenin at Ser552 is regulated through AKT activation due to PTEN targeted by miR-21 (38,39). Therefore, miR-21 activation of TCF-reporter activity might occur partially through the NF-κB/PI3K/AKT cascade.

In the present study, APC mutations in colorectal cancer patients were detected in the mutation cluster region. The majority of APC mutations in colorectal cancer occur in the mutation cluster region (40). The APC mutation rate in this study population (60 of 165, 36.4%) was lower than in other reports (~80%) (40–42). Immunohistochemical data indicated that nuclear β-catenin expression in colorectal tumours positively correlated with APC mutations and miR-21 expression.
levels (Table 1). Moreover, the highest prevalence of positive nuclear β-catenin expression was observed in colorectal tumours that had high miR-21 levels combined with APC mutation when compared with the other three subgroups (Table 1). These results are consistent with the cell model findings and showed that miR-21 overexpression may promote β-catenin nuclear localization in APC-mutated cells but not in APC-wild-type cells. Therefore, miR-21 levels may be an independent prognostic indicator in APC-mutated colorectal cancer but not in APC-wild-type colorectal cancer. However, this observation is not consistent with a previous report indicating that miR-21 levels may predict a poor outcome in all colon adenocarcinoma patients; this could be attributed to ~80% of Caucasian colon cancer patients having APC mutations (41,42).

In summary, we demonstrated that miR-21 may promote β-catenin nuclear translocation and β-catenin/TCF transcriptional activity and, in turn, enhance invasiveness in APC-mutated cells but not in APC-wild-type colon cancer cells. Correspondingly, APC-mutated colorectal cancer patients whose tumours have high miR-21 levels showed poorer OS and RFS compared with those with low miR-21 expression levels, but the prognostic value was not observed in APC-wild-type colorectal cancer patients.

Supplementary material
Supplementary Tables 1–6 and Figures 1–6 can be found at http://carcin.oxfordjournals.org/

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References

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