miR-216b promotes cell growth and enhances chemosensitivity of colorectal cancer by suppressing PDZ-binding kinase

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A R T I C L E   I N F O
Article history:
Received 21 March 2017
Accepted 30 March 2017
Available online xxx

Keywords:
Colonrectal cancer (CRC)  
PDZ-binding kinase (PBK/TOPK)  
miR-216b  
Oxaliplation (OXA)  
Chemosensitivity

A B S T R A C T
PDZ-binding kinase (PBK/TOPK) acts as oncogene in various cancers and correlates with drug response. However, few studies have examined the expression and roles of PBK in colorectal cancer (CRC). In this study, we found a significant increase in the expression of PBK in CRC tissues and cell lines. While overexpression of PBK promoted cell growth and decreased the toxicity effect of oxaliplation (OXA), targeting PBK with short hairpin RNA (shRNA) or novel PBK inhibitor HI-TOPK-032 effectively suppressed tumor growth and potentiated chemosensitivity in vitro and in vivo. Furthermore, there was a significant inverse correlation between the expressions of miR-216b and PBK. Further found that miR-216b could down-regulate PBK levels by binding to the 3’ untranslated region (3’UTR) of PBK. Notably, while miR-216b decreased cell proliferation and enhanced sensitivity of CRC cells to oxaliplation, re-expression of PBK dramatically reversed these events. Collectively, our data indicated that miR-216b may function as a tumor suppressor though regulating PBK expression, which provided promising targets and possible therapeutic strategies for CRC treatment.

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1. Introduction

Colonrectal cancer (CRC), also known as colon cancer, is one of the most prevalent cancer and the total incidence is keep rising [1]. Although many clinical advances have been achieved, the prognosis of patients with CRC is extremely poor [2]. The rates of treatment failure remain high due to resistance to multiple chemotherapeutic drugs [3,4]. Therefore, the in-depth study of the molecular mechanism of drug resistance is helpful to the treatment of malignant tumors including CRC.

PDZ-binding kinase (PBK/TOPK), a serine-threonine kinase, is highly expressed in various cancers, including lymphoma [5], breast cancer [6], and lung cancer [7]. Forced expression of PBK contributed to tumor development and metastasis, associating with poor prognosis [8,9]. Previous studies have revealed that PBK can interact with tumor suppressor p53 and regulate expression of transcriptional targets including p21 [10]. Moreover, PBK promoted tumor metastasis though regulating PI3K/PTEN/AKT pathway [11]. However, the regulatory mechanism of PBK expression in CRC is unknown.

MiRNAs, small non-coding RNA, have been reported as a new regulator of gene expression in recent years. MiRNAs perform multiply functions by affecting proliferation, migration and apoptosis [12]. An increasing number of studies have found that miRNAs act as oncogene or suppressor depending on tumor types, making them as early biomarkers [13]. MiR-216b has been considered to be a tumor suppressor in various cancers. Recent studies indicated that miR-216b inhibited cell proliferation and correlated with prognosis in hepatocellular carcinoma [14], breast cancer [15] and nasopharyngeal carcinoma [16]. Anther studies reported that miR-216b induced cell senescence in CRC [17]. However, the function roles of miR-216b in tumor growth and migration of CRC have not been clarified yet.

In this study, we found up-regulation of PBK in CRC tissues and cell lines, and high PBK expression was associated with poor overall survival. The effects of PBK in regulating cell growth and sensitivity to oxaliplation were evaluated in vitro and in vivo. An interaction between miR-216b and the 3’UTR of PBK was also found, and the regulation of PBK by miR-216b was confirmed, providing novel therapeutic targets for CRC treatment.

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http://dx.doi.org/10.1016/j.bbrc.2017.03.162
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Please cite this article in press as: J. Zou, et al., miR-216b promotes cell growth and enhances chemosensitivity of colorectal cancer by suppressing PDZ-binding kinase, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.03.162
2. Material and methods

2.1. Tissue specimens, cell lines and transfection

83 CRC samples and 43 normal colon tissues were obtained with medical informed consent. CRC cell lines (COLO-678, HT29, HT55, LS1034, SW1417, SW403, SW48, HCT8, COLO-205, LOVO, SW620, SW480, HCT116) and normal colon CCD-18Co (CRL-1459) cells were collected for ATCC. The cells were maintained in DMEM/RPMI 1640 (Hyclone) medium containing 10% FBS (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in humidified air at 37 °C with 5% CO2. Lipofectamine 2000 Reagent (Invitrogen, CA, USA) were used to transfect pre-miR-control (miR-co), pre-miR-216b (miR-216b), control shRNA (sh-co), shRNA specific for PBK (sh-PBK) into cells. Stable expression of PBK was achieved using PiggyBac (PB) transposon system, and selected with puromycin (5 μg/ml).

2.2. Quantitative RT-PCR (qRT-PCR)

TRizol Reagent (Invitrogen) was used to extract RNA from tissues and cells after treatment. The cDNA were obtained and then subjected to TaqMan microRNA assay (Applied Biosystems). GAPDH and U6 were used as endogenous control for mRNA and miRNA. Primers were obtained from PrimerBank, and the sequences of primers for PBK were as follows: forward 5'-CCA AAC ATT GTT GGT TAT CGT GC-3' and reverse 5'-GGC TGG CTT TAT ATC GTT CTT CT-3'.

2.3. Western blot and immunohistochemistry (IHC)

Cells were seeded in 6-well plates and cell protein mixtures were isolated using RIPA buffer with protease inhibitors (Sigma, St. Louis, MI, USA), and subjected to Western blot using primary antibody for PBK (1:1000) and GAPDH (1:1000) from Abcam (Cambridge, MA, USA). For IHC staining, samples were sliced into 4-μm sections, and the protein expression were measured by antibodies for PBK (1:250).

2.4. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 3000/well. After incubated overnight, cells were subjected to serum-free medium. Then cells were treated with 10 μM oxaliplation (OXA), or HI-TOPK-032 (5 μM or 10 μM). After treatment for an additional 4 d, the OD value were evaluated at 562 nm. The assays were repeated three times. In some experiments, cells were transected with shRNA or PB vector in advance.

2.5. In vivo studies

The flank of 8-week-old BALB/c female mice (Weitonglihua Biotechnology, Beijing, China) were used to establish xenograft model. 2 × 10^6 HT29 cells were injected into the BALB/c female mice. When tumors reached a size of 100 mm^3, mice were assigned into 4 groups (eight mice per group), and received oxaliplation (12.5 mg/kg) intraperitoneally once a day for 10 consecutive days.

![Fig. 1. PBK was up-regulated in CRC and correlated with poor prognosis.

(A) Quantitative RT–PCR analysis (qRT-PCR) was performed to examine the expression of PBK in 43 normal colon tissues (NT) and 83 colorectal cancer (CRC) samples.

(B) The expression of PBK in normal colon and colorectal cancer (CRC) tissues was evaluated by IHC staining, and the representative graphs were shown. Scale bar, 50 μm.

(C) The overall survival (OS) of patients with high (n = 43) and low (n = 37) levels of PBK was compared using mantel-Cox log-rank test. P = 0.0170.

(D) A panel of CRC cell lines and normal colon cell line CCD-18Co together with several normal tissues were selected to evaluate the expression of PBK.](http://dx.doi.org/10.1016/j.bbrc.2017.03.162)
HI-TOPK-032 (5 mg/kg) or vehicle was injected 3 times per week for 20 days. Tumor volumes were measured every 4 days using caliper measurements and calculated by the formula \( \pi \text{length} \times \text{width}^2 / 6 \).

2.6. Luciferase activity assay

The luciferase activity assay was performed to determine the interaction between miR-216b and PBK. Both wild type (WT) or mutant (Mut) of PBK 3’UTR and pre-miR-216b (miR-216b) or pre-miR-control (miR-co) were transfected into 293T cells using Lipfectamine 2000 (Invitrogen). 48 h after transfection, relative luciferase activity was measured using a Dual-Luciferase system (Promega, Madison, Wisconsin).

2.7. Statistical analysis

All data are presented as mean ± s.e.m. Two-tailed t-test or one-way ANOVA were used for analysis of differences between groups. Statistical analyses were performed using GraphPad Prism program version 5 (GraphPad Software, USA), with \( P \) values less than 0.05 considered statistically significant.

3. Results

3.1. PBK was up-regulated in CRC and correlated with poor prognosis

To explore the biological function roles PBK in CRC, we first analyzed the levels of PBK in 43 normal colon and 83 CRC tissues. As shown in Fig. 1A, PBK was significantly up-regulated in CRC than that in normal colon tissues (\( P < 0.001 \)). IHC staining was performed to verify the expression of PBK in CRC tissues (Fig. 1B). From analysis of the overall survival of patients, we found that sufferers with high level of PBK shared poorer outcome than those with low level of PBK (Fig. 1C). Furthermore, we also found a up-regulation in the expression of PBK in a panel of CRC cell lines (Fig. 1D).

3.2. PBK regulated tumor growth and chemosensitivity of CRC

To further examine the roles of PBK in CRC, we modified the expression of PBK in CRC cell lines by transfection with PBK

![Fig. 2](image-url)

Fig. 2. PBK regulated tumor growth and chemosensitivity of CRC.
(A) HT29 and HCT8 cells were transfected with empty control PiggyBac (PB) transposon system (PB-co) or PBK expressing vector (PB-PBK), and selected with puromycin (5 \( \mu \)g/ml), then the PBK expression was evaluated by Western blot (upper). Silencing PBK by transfection with PBK specific shRNA was also performed (bottom).
(B) After transfected with PB-co or PB-PBK vector, HT29 and HCT8 cells were then treated with 10 \( \mu \)M oxaliplation (OXA), and the relative proliferation of HT29 and HCT8 cells were examined by analysis of OD value at 562 nm.
(C) The relative proliferation was examined in PBK silencing cells in presence of oxaliplation (OXA, 10 \( \mu \)M).
(D) HT29 cells were treated with PBK inhibitor HI-TOPK-032 (5 \( \mu \)M or 10 \( \mu \)M), then the effects of HI-TOPK-032 on cell proliferation were examined at 0 h, 24 h, 48 h, 72 h.
(E) After treatment with oxaliplation (OXA, 10 \( \mu \)M) or/and HI-TOPK-032 (5 \( \mu \)M), the growth of HT29 cells was examined.
(F) After inoculation with HT29 cells, mice were treated intraperitoneally with oxaliplation (12.5 mg/kg), HI-TOPK-032 (5 mg/kg) or their combination. The tumor volume was calculated every 4 days, \( n = 10 \).
expressing vector (PB-PBK) or PBK specific short hairpin RNA (sh-PBK). The transfection efficiency was confirmed by Western blot in HT29 and HCT8 cells (Fig. 2A). As shown in Fig. 2B and C, while forced expression of PBK accelerated tumor cell growth, a great inhibition on cell growth were observed in PBK-depleted cells. We further examined the effects of PBK on the chemosensitivity of CRC cell lines. While oxaliplation (OXA) markedly impaired cell growth, excessive expression of PBK significantly decreased the anti-tumor effect of oxaliplation (Fig. 2B and C), indicating that overexpression of PBK may mediate resistance to oxaliplation in CRC cells. HI-TOPK-032, an inhibitor of PBK, was further used to examine the effects of PBK on tumor growth. As shown in Fig. 2D, the OD value were decreased in a dose-dependent manner after treated with PBK inhibitor in CRC cells. While the proliferation of CRC cells was moderately suppressed by oxaliplation or HI-TOPK-032, a combination of them achieved more intense anti-tumor effects than each drug alone (Fig. 2E). These results were further confirmed in vivo using a HT29 colorectal xenograft model (Fig. 2F).

3.3. PBK was a target of miR-216b

We further found that miR-216b was down-regulated in CRC tissues, compared with that of normal tissues (Fig. 3A). Interestingly, there was an remarkable inverse relation between the levels of PBK and miR-216b in CRC tissues ($r = -0.5169, P < 0.01$; Fig. 3B).

To validate the association between PBK and miR-216b, we firstly performed the prediction using Targetscan (http://www.targetscan.org/). As shown in Fig. 3C, miR-216b was complementary to the 3’untranslated region (3’-UTR) of PBK. We further transfected the wild type (WT) and mutant (Mut) of PBK 3’-UTR into 293T cells together with miR-216b. It was demonstrated that the luciferase activity was reduced when co-transfection with wide type PBK 3’-UTR and miR-216b (Fig. 3D), suggesting miR-216b could directly bind to the 3’UTR of PBK. Similar results were found in HT29 and HCT8 cells (Fig. 3E). After transfected with miR-216b, an obvious decrease in the expression of PBK was found in both HT29 and HCT8 cells (Fig. 3F). Conversely, the levels of PBK were enhanced in miR-216b-depleted cells, suggesting the regulation of PBK by miR-216b in CRC cells.

3.4. miR-216b inhibited cell growth and enhanced chemosensitivity by regulating PBK

Since miR-216b was down-regulated in CRC and could suppress the expression of PBK, we further examined the roles of miR-216b in CRC. Our results showed that miR-216b markedly inhibited the proliferation of both HT29 and HCT8 cells (Fig. 4A), whereas treatment with miR-216b inhibitor (anti-miR-216b) led to a strong enhancement in cell growth (Fig. 4B). We further studied whether PBK was involved in miR-216b mediated anti-tumor responses. It

![Fig. 3. PBK was a target of miR-216b.](image-url)

(A) The levels of miR-216b between 43 normal colon tissues (NT) and 83 colorectal cancer (CRC) samples, $P < 0.001$.
(B) The correlation between PBK and miR-216b in CRC tissues was examined ($r = -0.5169, P < 0.01$).
(C) The sequences of miR-216b and its potential binding site in 3UTR of PBK were shown. Vector containing the wild type and corresponding mutant of PBK 3’UTR were shown.
(D) After transfected with pre-miR-216b (miR-216b) and PBK 3’UTR vector, the luciferase activity was measured in 293T cells.
(E) Luciferase activity assay was further performed in both HT29 and HCT8 cells.
(F) HT29 and HCT8 cells were transfected with miR-216b-expressing vector (miR-216b), or its inhibitor (anti-miR-216b), or their corresponding controls, and the expression of PBK was examined by Western blot.
demonstrated that restoration of PBK expression could dramatically reverse miR-216b-mediated growth suppression of HT29 and HCT8 cells (Fig. 4C). Furthermore, the anti-miR-216b-enhanced cell growth can be partly vanished though silencing PBK (Fig. 4D). To further validate the effects of miR-216b on oxaliplation-based chemotherapy, we assessed the proliferation ability of miR-216b overexpressing cells in presence of oxaliplation. The cytotoxicity of oxaliplation was expanded when transfection with miR-216b, however, these events were further abolished by re-expression of PBK (Fig. 4E). Collectively, these data indicated that miR-216b suppressed tumor growth and cemented chemosensitivity to oxaliplation though targeting PBK.

4. Discussion

Many researches have reported that PBK may act as oncogene and contribute to cancer growth and anti-apoptotic effects in various cancers, making it a potential therapeutic target [18,19]. Several PBK inhibitors, such as HI-TOPK-032 and OTS514, have been developed and displayed effective anti-tumor effects in vitro and in vivo [20,21]. In this study, we examined the levels of PBK in CRC tissues and cell lines. Interestingly, we found PBK was up-regulated in CRC tissues, compared to normal colon tissues (Fig. 1A and B). In addition, PBK was further proved to serve as an indicator of poor prognosis (Fig. 1C), consistent with the previous report in lung cancer [11]. While restoration of PBK contributed to tumor growth, knockdown of PBK led to substantial decrements in proliferation capacity (Fig. 2), suggesting that PBK may act as a useful prognosticator for tumor development.

A variety of chemotherapeutic drugs, such as oxaliplation (OXA), are extensively used for the management of advanced or metastatic colorectal cancer [22]. However, a subset of patients still obtained resistance to chemotherapy. The strong and consistent over-expression of PBK in CRC facilitated us to verify whether PBK was associated with drug resistance. As expected, forced expression of PBK appeared to suppress the anti-tumor effects induced by oxaliplation (Fig. 2B), suggesting that PBK may act as a resistance gene and induce resistance to chemotherapy in CRC. It has demonstrated that PBK induced resistance to EGFR tyrosine kinase inhibitors by activating c-Jun in lung cancer [23]. Recent reports said that PBK could affect doxorubicin-mediated apoptosis by regulating p53 activity and p21 expression [10]. The detailed resistance mechanisms mediated by PBK in CRC still need further research. Moreover, our data indicated that genetic or pharmacological inhibition of PBK dramatically enhanced the drug response of oxaliplation, suggesting that PBK emerged as a potential candidate therapeutic target for CRC.

Encouraging evidences have showed that a number of miRNAs were correlated with gene expression and cellular functions, including cell proliferation and differentiation [24]. In this study, we found miR-216b was down-regulated in CRC tissues and cell lines (Fig. 3A), and associated with prognosis of patients (Fig. 3B), suggesting miR-216b may play dominant role in CRC treatment. Actually, miR-216b has been reported as tumor suppressor recently. Forced expression of miR-216b suppressed cell proliferation and miR-216 mediated multi-drug resistance in hepatocellular carcinoma [25,26]. We also demonstrated that miR-216b regulated PBK expression though directly binding to the 3’UTR of PBK (Fig. 3C).

Fig. 4. miR-216b inhibited cell growth and enhanced chemosensitivity by regulating PBK.

(A) After transfection with control miRNA (miR-co) or pre-miR-216b (miR-216b), the relative proliferation was detected in HT29 and HCT8 cells.

(B) After treatment with miR-216b inhibitor (anti-miR-216b), the growth abilities of HT29 and HCT8 cells were examined.

(C) After transfected with miR-216b and PBK expression vector (PB-PBK), the proliferation assays were performed in HT29 and HCT8 cells.

(D) After transfected with anti-miR-216b and sh-PBK vector, the proliferation assays were performed in HT29 and HCT8 cells.

(E) After transfected with miR-216b or/and PB-PBK, HT29 cells were subsequently treated with 10 μM oxaliplation (OXA). The proliferation of these cells were examined.

Please cite this article in press as: J. Zou, et al., miR-216b promotes cell growth and enhances chemosensitivity of colorectal cancer by suppressing PDZ-binding kinase, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.03.162
Luciferase assays and Western blot further confirmed the relation between PBK and miR-216b. We found that forced expression of miR-216b repressed tumor growth and enhance chemotherapy synergy (Fig. 4A), accompany with decrease of PBK expression as shown in Fig. 3F. Notably, PBK re-expression reversed miR-216b induced growth inhibition, indicating that miR-216b dominated shown in Fig. 3F. Notably, PBK re-expression reversed miR-216b dominated cell growth and drug response by breaking PBK associated pathways.

Taken together, our study showed an interesting axis consisted of miR-216b and PBK regulating tumor proliferation and chemosensitivity of CRC. Knockdown of PBK inhibited cell growth and strengthen anti-tumor effects of oxaliplation-based treatment. Our data provided evidence that miR-216b-PBK axis could be a crucial molecular drug target for CRC therapy.

Acknowledgments

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.03.162.

References
