**AC1MMYR2 impairs high dose paclitaxel-induced tumor metastasis by targeting miR-21/CDK5 axis**

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**Abstract**

Paclitaxel (taxol) is a widely used chemotherapeutic agent for many solid tumors, while continual taxol treatment is revealed to stimulate tumor dissemination. We previously found that a small molecule inhibitor of miR-21, termed AC1MMYR2, had the potential to impair tumorigenesis and metastasis. The aim of this study was to investigate whether combining AC1MMYR2 with taxol could be explored as a means to limit tumor metastasis. Here we showed that abnormal activation of miR-21/CDK5 axis was associated with breast cancer lymph node metastasis, which was also contributory to high dose taxol-induced invasion and epithelial-mesenchymal transition (EMT) in both breast cancer cell line MDA-MB-231 and glioblastoma cell line U87VIII. AC1MMYR2 attenuated CDK5 activity by functional targeting CDK5RAP1, CDK5 activator p39 and target p-FAKser732. A series of in vitro assays indicated that treatment of AC1MMYR2 combined with taxol suppressed tumor migration and invasion ability in both MDA-MB-231 and U87VIII cell. More importantly, combination therapy impaired high-dose taxol induced invadopodia, and EMT markers including β-catenin, E-cadherin and vimentin. Strikingly, a significant reduction of lung metastasis in mice was observed in the AC1MMYR2 plus taxol treatment. Taken together, our work demonstrated that AC1MMYR2 appeared to be a promising strategy in combating taxol induced cancer metastasis by targeting miR-21/CDK5 axis, which highlighted the potential for development of therapeutic modalities for better clinic taxol application.

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**Introduction**

Metastatic disease is found in 40% of epithelial cancer patients and becomes the major cause of cancer-related death [1–3]. Paclitaxel (taxol), as a microtubule-stabilizing agent, is widely used to treat patients with many forms of cancer [4]. However, continued taxol treatment was revealed to induce the epithelial–mesenchymal transition (EMT) and promoted lung metastasis formation in animals bearing leukemic cells [5]. A recent report indicated that 2 μM taxol, an achievable clinical dose, could increase the invadopodia formation [6]. Consequently, a better understanding of the underlying molecular mechanisms will facilitate the development of effective therapeutic strategies for patients to prevent incipient metastasis in the chronic taxol treatment.

MicroRNAs (miRNAs) are a class of small non-coding RNAs, acting like gene expression master for many important biological pathways [7–11]. It has been confirmed that the overexpression of miR-21 correlated with advanced breast cancer stages, lymph node metastasis, and poor survival in patients [12,13]. Several studies demonstrated that anti-miR-21 therapy could be a powerful strategy for the regulation of tumor metastasis [14–17]. Our previous study demonstrated that AC1MMYR2, a specific small-molecule inhibitor of miR-21, was capable of suppressing tumor invasion and realizing of EMT reversal both in vitro and in vivo [18]. However, it is not clear whether AC1MMYR2 can tune to orchestrate the high dose taxol induced EMT and metastasis.

In this study, we found that treatment with taxol activated miR-21/CDK5 axis, which contributed to EMT and migration in both breast cells lines MDA-MB-231 and glioblastoma U87VIII [19,20].
AC1MMYR2 attenuated CDK5 activity by elevating miR-21 biological target CDK5RAP1, decreasing the CDK5 activator p39 and its downstream target p-FAK as expression. Taxol treatment-enhanced invadopodia formation, cell migration and invasion were significantly inhibited via using AC1MMYR2. Moreover, EMT marker β-catenin and vimentin were dramatically decreased in AC1MMYR2 combined with taxol treatment. Most important, a significant reduction of lung metastasis in orthopic breast cancer transplantation mice model was observed in the AC1MMYR2 plus taxol treatment. Therefore, AC1MMYR2 may be translated into clinic as an effective therapeutic strategy for patients to prevent taxol treatment induced metastasis. Collectively, AC1MMYR2 might be a potential neoadjuvant treatment with taxol in highly invasive cancers.

Materials and methods

Cell lines and culture conditions

Human breast cancer cells MDA-MB-231 were purchased from American Type Culture Collection (ATCC). U87-MG cells with EGR-VIII stable transfection (U-87 MG EGR-VIII, U87VIII) were provided by Huan Ren, Department of Immunology, Harbin Medical University [21]. Both of the two highly invasive cancer cells were cultured at 37°C in a 5% CO2 humidified incubator and maintained in DMEM supplemented with 10% fetal bovine serum and 5% penicillin–streptomycin.

Cell fixation and staining

Cells were fixed for 15 min, then incubated with 0.1% Triton-X 100, followed by blocking with 5% BSA for 30 min. Immunofluorescence staining was conducted using antibodies against p-39, β-catenin, tubulin-alpha, and E-cadherin (1:1000 dilutions; cell signaling technology), and the cells were visualized using FITC-1000 laser scanning confocal microscopes. For invadopodia formation detection, cells were stained with anti-cortactin antibody and Alexa Fluor 568-phalloidin for detection of invadopodia formation. Quantification of cells with invadopodia was performed in three independent experiments in which at least 300 cells were counted.

RNA extraction real time PCR

Cells were treated with various dose of taxol for 48–72 h. Then, the RNA of different groups was extracted using a trizol reagent (Invitrogen) according to the standard protocol. The real-time PCR protocol was carried out with the miVanaq RT-PCR miRNA detection kit (Ambion). Amplification reaction was performed using MJ-real-time PCR (Bio-Rad, Hercules, CA, USA) and the protocol was performed for 40 cycles at 95°C for 3 min, 95°C for 15 s, and 60°C for 30 s. U6 was used as the internal control.

Western blotting

Cell lysates were separated in an analytical 10% SDS-PAGE gel and transferred onto the polyvinylidene fluoride membrane. Antibodies against human CDK5 p39, phospho-FAK, CDK5RAP1, E-cadherin and β-actin were used as primary antibodies. Rabbit or mouse IgG antibody coupled with horseradish peroxidase was used as a secondary antibody. The density of the immunoblots was determined by Image J analysis software.

Matrigel invasion assay

Cells with serum-free DMEM were seeded in the upper chambers (Becton Dickinson) for 24 h at 37°C. And medium in the lower chamber was replaced with 10% fetal bovine serum and 5% penicillin–streptomycin. As a secondary antibody. The density of the immunoblots was determined by Image J analysis software.

Orthotopic nude mouse models and treatment

BALB/c nude mice at 4 weeks of age were purchased from the Animal Center at the Cancer Institute at Chinese Academy of Medical Science (Beijing, China). To establish orthotopic models, 2 × 106 MDA-MB-231 cells transduced with luciferase lentivirus were injected into mammary fat pads of each nude mouse. Mice were randomized assigned to four groups (n = 8 per group). Seven days after implantation, DMSO, AC1MMYR2 (25 mg/kg), taxol (5 mg/kg) alone or in combination was administered by intraperitoneal injection every 3 days for 42 days. Mice were imaged for Fluc activity once a week. On day 42, the mice were killed, and mice hearts, lungs, livers, spleens, and kidneys were removed for luminescent imaging.

Hematoxylin and eosin staining and immunohistochemistry analysis

The paraffin-embedded tissue sections were used for hematoxylin and eosin (H&E) staining and immunohistochemistry analysis as previous described [22]. The sections were incubated with primary antibodies (1:100 dilutions) overnight at 4°C, followed by a biotin-labeled secondary antibody (1:100 dilutions) for 1 hour at 37°C and then incubated with ABC-peroxidase and 3,3-diaminobenzidine, counterstained hematoxylin and visualized using a light microscope.

Statistical analyses

SPSS 16.0 (SPSS) was used for all calculations. All data are represented by the mean ± SD. Statistical significance was determined at *P < 0.05, ** P < 0.01.

Results

Expression of miR-21 and CD5 associated with lymph node metastasis in breast cancer patients

According to the TCGA data, both miR-21 and CD5 are elevated in 1170 breast tumors samples compared with normal tissues (Fig. 1A), which is consistent with previous reports [12]. The expression pattern of miR-21 and CD5 were further examined in 58 pairs of breast carcinoma tissues with metastasis or not. The confocal microscopy results revealed that stronger miR-21 staining was observed in invasive carcinoma samples compared with the non cases (Fig. 1B). CD5 were expressed in 39 of 39 invasive carcinoma samples tested (Fig. 1C). However, only 7 of 19 non-lymph node metastasis samples expressed CD5. These results strongly indicated that expression of miR-21 and CD5 were elevated in breast cancer tissues and associated with tumor metastasis.

High dose taxol induced miR-21/CD5 activation and EMT

Taxol is widely used in cancer chemotherapy, while continual treatment is revealed to induce metastasis. In this study, we first examined the effect of drug on EMT. As shown in Fig. 2A, increased level of E-cadherin was detected in low dose taxol (0.5 μM) treatment, which played a critical role in the EMT. However, in the high dose taxol group, with the drug concentration of 2 μM, E-cadherin was substantially decreased, while expression of β-catenin and vimentin were dramatically increased, indicating the activation of EMT. Moreover, cells exhibited significantly elevated levels of miR-21 and CD5 in high dose taxol treatment (Fig. 2B). These result suggested that high dose taxol induced EMT in a miR-21/CD5 dependent manner.

AC1MMYR2 inhibited taxol-induced cell migration and invasion ability

Our previous study demonstrated that AC1MMYR2, a novel small molecule inhibitor of miR-21, was capable of suppressing tumor invasion and realizing of EMT reversal both in vitro and in vivo [18]. Next, cell migration and invasion ability were evaluated by AC1MMYR2 plus taxol treatment in both breast cancer cell line MDA-MB-231 and glioblastoma cell line U87VIII. Cells were stained for cortactin and F-actin, and co-localization was observed at the cell periphery and in invadopodia as punctate dots. Taxol treatment significantly enhanced invadopodia number in MDA-MB-231 cells, increased almost 2–3 fold compared with the control group (Fig. 3A), which was consistent with previous study [6]. AC1MMYR2 dramatically reduced the number of cells capable of forming invadopodia; only a few invadopodia were observed in AC1MMYR2 plus taxol group (Fig. 3B and C). Furthermore, in taxol treated cells, F-actin showed a stronger cortical pattern than in control cells, suggesting strong mesenchymal features and high movement ability. While in AC1MMYR2 or combined with taxol treated cells, F-actin showed
relative stress fiber pattern, de-polymerization and redistribution, indicating that the formation of F-actin bundles was suppressed.

Moreover, high dose taxol treatment resulted in noticeable increased cell invasion number and migration ability, as shown in Fig. 3D and E. After the addition of AC1MMYR2, cell invasion number was decreased about 45–55%, compared with the taxol treatment alone. In addition, taxol plus with AC1MMYR2 treated cells failed to fill the gap 48 hours after the scratch, suggesting that directional migration was impaired by the combination therapy (Fig. 3F and G). These data strongly suggested that AC1MMYR2 was capable of inhibiting high dose taxol-induced invasion and metastasis.

CDK5 was required for taxol-induced metastasis

To further explore the mechanism of AC1MMYR2 mediated cell metastasis, RNA sequence was employed to identify the potential biological target of miR-21 that was up-regulated by AC1MMYR2. GO analysis for the biological processes was conducted on the 534 genes whose expression was significantly altered in response to 24 h treatment with AC1MMYR2 in both U87VIII and MDA-MB-231 cells. Meanwhile, KEGG pathway analysis revealed that there were 14 different pathways corresponding to the target genes. And only one target gene was found in both cells, that is CDK5RAP1 (CDK5 regulatory subunit associated protein 1), according to the mRNA sequencing data (Fig. 4A). CDK5RAP1 was discovered to inhibit the active CDK5 kinase, which is a member of the cyclin-dependent kinase family of proline-directed protein kinases [23,24]. A recent study unraveled a novel function of CDK5 as a mediator of EMT and migration in cancer cells, as well as in invadopodia formation, implicating it as a potential target for prevention of tumorigenesis and metastasis [25–27]. As shown in Fig. 4B, taxol resulted in significant increased expressions of CDK5, and downstream target, p-FAK<sup>Y739</sup>. While both of the two protein levels were decreased in the cells treated with AC1MMYR2 or combined with taxol. Moreover, CDK5RAP1 expression was dramatically increased.

To further confirm whether CDK5 was a critical mediator of AC1MMYR2 blocked high dose taxol induced metastasis, CDK5 was stably knocked down or over-expressed in cancer cells. The shRNA-mediated CDK5 silencing was able to inhibit the taxol induced EMT in both U87VIII and MDA-MB-231 cancer cells. As shown in Fig. 4C, increased E-cadherin expression was detected, while expressions of mesenchymal markers β-catenin and vimentin were significantly decreased in the combination therapy, which suggested that CDK5 knockdown had a potential synergistic effect on drug-induced EMT reversal. Thus, our loss-of-function study also pointed to a critical role of CDK5 in taxol-induced EMT. Conversely, CDK5

Fig. 1. miR-21 and CDK5 associated with tumor metastasis. (A) The expression of miR-21 and CDK5 was analyzed in 1170 breast cancer samples supplied by TCGA database. (B) MiR-21 and CDK5 expression in invasive breast carcinoma and non-lymph node metastasis case were detected by in situ hybridization and IHC, respectively.

Fig. 2. High dose taxol induced EMT and miR-21/CDK5 activation. Evaluation of E-cadherin, vimentin and β-catenin expression (A) and miR-21 level (B) in cells treated with various dose of taxol.
Fig. 3. AC1MMYR2 reversed taxol-induced invadopodia formation and invasion. Cells were treated with taxol, AC1MMYR2, or the combination of taxol and AC1MMYR2. (A) Fixed cells were stained for anti-cortactin (green) and F-actin (phalloidin, red). Representative invadopodia are highlighted with white arrows (the colocalization of anti-cortactin and phallolidin indicated by a yellow color inside the cells); scale bar: 20 μm. (B) Quantitation of invadopodia formation in U87VIII cells and MDA-MB-231 cells (C) For each measurement, ≥10 fields of view with ≥100 cells were analyzed; *P < 0.05, **P < 0.01 according to Student’s t-test. (D) Transwell invasion assays. Cells were placed into the upper wells of a transwell plate, and FBS in serum-free medium was added to the lower wells. After 48 h of incubation, the percentage of invaded cells was calculated. (E) Wound-healing assays in U87VIII and MDA-MB-231 cells (F). The migration distance on the cell monolayer was measured 24 h after the scratches were made. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. AC1MMYR2 impairment of taxol-induced EMT is dependent on CDK5 activity. (A) Bioinformatics analyses indicated that CDK5RAP1 was the only biological target of miR-21 in both the U87VIII and MDA-MB-231 cell lines. (B) CDK5 pathway-related proteins were analyzed using Western blotting and normalized to GADPH. Taxol enhanced the expression of CDK5, CDK5RAP1 and p-FAKser732 in both U87VIII and MDA-MB-231 cells, whereas these increases were abrogated by cotreatment with AC1MMYR2. (C) Expression of the EMT-related markers E-cadherin, β-catenin and vimentin was evaluated using western blotting in cells transfected with either anshCDK5 lentivirus or a CDK5 overexpression plasmid. Lenti virus-mediated CDK5 silencing was able to inhibit taxol-induced EMT in both U87VIII and MDA-MB-231 cancer cells. Moreover, the overexpression of CDK5 blocked the function of AC1MMYR2. (D) E-cadherin, N-cadherin, β-catenin, and tubulin-α expression levels and subcellular location were confirmed using immunofluorescence staining. Scale bar: 20 μm.
overexpression blocked the AC1MMYR2 performing function. The Western blot showed that the combination therapy-induced increase in E-cadherin expression was reduced, which suggests that CDK5 activity determines (or is at least a critical mediator of) the function of AC1MMYR2 in high dose taxol induced EMT. Furthermore, stronger staining of E-cadherin was observed in the AC1MMYR2 plus taxol treated U87-VIII cells, with decreased nucleus distribution of β-catenin, and weak fluorescence of N-cadherin (Fig. 4D), which further confirmed our result. In addition, taxol group stabilized much more intact microtubules, which is required for invadopodia formation [28,29]. However, AC1MMYR2 alone or combined with taxol induced tubulin-a rearrangement and inhibited both rates and extent of tubulin-a polymerization.

AC1MMYR2 regulated CDK5 kinase activity

We further explored the mechanism of AC1MMYR2 mediated CDK5 activity. The transcription factor Egr-1, containing the binding site of the CDK5 promoter region, had been validated to regulate CDK5 activity [30]. Fig. 5A showed that CDK5RAP1 and Egr-1 expression was dramatically enhanced after AC1MMYR2 treatment independently or in combination with taxol.

To be enzymatically active, CDK5 must form a complex with one of two known regulatory subunits, p35 and p39, both of which are expressed at high levels in cancer [31]. High dose taxol-mediated increase in CDK5 expression was decreased by AC1MMYR2 treatment with a concomitant reduction in p39 expression. To further determine whether and how AC1MMYR2 regulates the activation of CDK5, co-immunoprecipitation was performed using CDK5 or p39 antibodies, respectively. The Western blot in Fig. 5B shows that CDK5 bound to p39 in untreated cells, and this association ceased in AC1MMYR2 treatment. Moreover, nucleus staining of p39 significantly decreased after AC1MMYR2 treatment in both U87VIII and MDA-MB-231 cells (Fig. 5C and 5D), which is further confirmed by Western blot. These observations suggested that AC1MMYR2 suppressed CDK5 kinase activity through elevating CDK5RAP1 and Egr-1 activity and blocking the interaction of its activator, p39.

AC1MMYR2 suppressed tumorigenesis and reduced taxol-induced lung metastasis in vivo

Next, we used a nude mouse orthopic tumor transplantation model to investigate the role of combination therapy in tumor metastasis in vivo. Taxol suppressed tumor growth to some extent, compared with the PBS group (Fig. 6A–C). However, more lung metastasis fluorescent signal was observed at the termination of the study (Fig. 6D). AC1MMYR2 combined with taxol group significantly reduced the taxol-induced lung metastasis, which is further evidenced by HE staining (Fig. 6E). Furthermore, the ability of tumorigenesis caused by the injection AC1MMYR2 was significantly lower than that of the control cells, as manifested by the apparent smaller tumor size (Fig. 6B and C).

Fig. 5. AC1MMYR2 regulates CDK5 activity. (A) Western blot assays of CDK5RAP1, Egr-1 and p39 expression levels after AC1MMYR2 alone or in combination with taxol. GAPDH was used as the loading control. (B) Cell lysates were used to immunoprecipitate CDK5 or p39 with specific antibodies. The whole immunoglobulin (IgG) was used as a control antibody. The immunoprecipitated complexes were subjected to western blot analysis with specific antibodies against CDK5 and p39 as indicated. P39 subcellular location in U87VIII (C) and MDA-MB-231 (D) was detected using confocal microscope. Scale bar: 20 μm.
Furthermore, the expression levels of CDK5 and β-catenin were significantly decreased in tumor specimens from the combination therapy group (Fig. 6E), which is consistent with our result in vitro and further, verifying our hypothesis.

**Discussion**

Due to the heterogeneity of cancer cells, a single-drug therapy often results in the emergence of drug resistance and tumor metastasis. Our work is the first to report that single taxol treatment induced EMT and lung metastasis by CDK5 dependent miR-21 up-regulation. Niu’s group demonstrated that single Dox therapy induced NF-κB-dependent microRNA-21 up-regulation and promoted breast cancer cell invasion [17]. Therefore, chemotherapy combined with microRNA gene therapy becomes a powerful strategy in cancer therapy.

MicroRNAs (miRNAs) are a class of small noncoding RNAs, which primarily bind to the 3′-untranslated region (3′-UTR) of target mRNA and negatively regulate gene expression at the post-transcriptional level [10,11]. As the key member of onco-microRNA family, miR-21 overexpression was frequently found in various solid tumors, thereby facilitating the initiation of EMT, which make miR-21 a promising therapeutic target in cancer therapy [32,33]. Our previous studies demonstrated that AC1MMYR2, the small molecule targeting miR-21, contributed to suppression of the tumor growth and metastasis [18]. In this study, we further pointed out that AC1MMYR2 could also successfully reverse taxol-induced metastasis and EMT.
Our mechanistic study revealed that AC1MMYR2 reversed high dose taxol induced EMT by attenuating CDK5 activity (Fig. 7). Unlike the other Cdk5, CDK5 has been shown to strengthen cell-to-matrix adhesion and regulate migration, which is activated by one of two non-cyclin proteins, p35 or p39. High dose taxol elevated CDK5 expression and promoted cell migration. Moreover, knocking down CDK5 impaired taxol induced EMT. Following AC1MMYR2 treatment combined with taxol, CDK5RAP1 expression, which was predicted as the miR-21 downstream target, was significant elevated; in addition, decreased p39 expression and nuclear p39 distribution was observed, thereby resulting in the attenuation of CDK5 activity and downstream target gene expression, including p-FAKSer732 and β-catenin.

Further investigation revealed that the pro-survival mechanism mediated by the CDK5 complex is accomplished by activation of focal adhesion kinase (FAK) pathways. FAK is involved in many aspects of the metastatic process and thus, overexpression hyper phosphorylation activity of FAK has been reported in a variety of human cancers [34,35]. P-FAKSer732, which was been identified as a target of CDK5, demonstrated to be implicated in microtubule organization and cell migration [36]. Cell–cell adhesion signaling molecule β-catenin was also identified as a CDK5 complex-interacting protein [37]. The loss of E-cadherin can lead to the accumulation of β-catenin, and stabilized β-catenin in the cytoplasm translocates to the cell nucleus, where it forms a β-catenin-TCF/LEF transcriptional complex and induces the transcription of target genes implicated in EMT and carcinogenesis [38,39]. In the present study, our results confirmed that these genes were the mediators of high dose taxol effects on cell invasiveness and EMT. Remarkable decreased p-FAKSer732 expression and nucleus β-catenin distribution was detected in AC1MMYR2 combined taxol group. When tested in animals, AC1MMYR2 suppressed the growth of human breast cancer metastasis in the lung. We found a significant reduction of lung metastasis in mice treated with AC1MMYR2 plus taxol. Immunohistochemistry results revealed that taxol induced CDK5, p-FAKSer732, and nucleus β-catenin expression, and treatment of animals with AC1MMYR2 suppressed the expression of all the gene products.

Conclusions

So far, only few examples of small molecules targeting oncogenic miRNAs have been reported. Our work demonstrated that AC1MMYR2 appeared to be a promising strategy in combating taxol induced metastasis, which highlighted the potential for development of therapeutic modalities for better clinic taxol application.

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Conflict of interest

None.

References

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