Overexpression of miR-22 reverses paclitaxel-induced chemoresistance through activation of PTEN signaling in *p53*-mutated colon cancer cells

Jian Li · Yangde Zhang · Jingfeng Zhao · Fangren Kong · Yuxiang Chen

Abstract Chemoresistance is a key cause of treatment failure in colon cancer. MiR-22 is a tumor-suppressing microRNA. To explore whether miR-22 is an important player in the development of chemoresistance in colon cancer, we overexpressed miR-22 and subsequently tested its role in cell proliferation, apoptosis, survival, and associated signaling in *p53*-mutated HT-29 and HCT-15 cells, and *p53* wild-type HCT-116 cells. We further investigated the role of miR-22 on cytotoxicity of paclitaxel in both the *p53*-mutated and *p53* wild-type colon cancer cells. Results showed that HT-29 and HCT-15 cells were resistant to paclitaxel-induced cytotoxicity, which normally inhibits cell proliferation and survival, and induces apoptosis. Conversely, HCT-116 was relatively sensitive to the cytotoxicity of paclitaxel. Overexpression of miR-22 significantly decreased cell proliferation and survival, and induced cell apoptosis in the *p53*-mutated colon cancer cells, but played no role in the *p53* wild-type cells. Importantly, miR-22 overexpression enhanced the cytotoxic role of paclitaxel in *p53*-mutated HT-29 and HCT-15 cells, but not in *p53* wild-type HCT-116 cell. We further demonstrated that the tumor-suppressive role of miR-22 in *p53*-mutated colon cancer cells was mediated by upregulating PTEN expression, which negatively regulated Akt phosphorylation at Ser^{473} and MTDH expression, and subsequently increased Bax and active caspase-3 levels. Our study is the first to identify the tumor-suppressive role of miR-22 and its associated signaling in the *p53*-mutated colon cancer cells and highlighted the chemosensitive role of miR-22.

Keywords Colon cancer · Chemoresistance · miRNA · Apoptosis · Paclitaxel

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Introduction

Reports from the World Health Organization (2009) indicate that there are 655,000 annual deaths worldwide from colorectal cancer (CRC). CRC is the fourth most common cancer in the United States and is ranked third among cancers in the western world [1]. The incidence of CRC in China is relatively lower, but has shown an increased prevalence in recent years [2]. In general, the stage of the tumor dictates the possible treatment strategies for CRC in patients. Early stages of CRC are operable and surgery is the primary treatment. Chemotherapy remains the adjuvant treatment after surgery only in cases where cancer has spread to the lymph nodes. However, many CRCs are detected at later stages and chemotherapy becomes the main option to reduce metastasis, shrink tumor size, or slow tumor growth [3].

Even with high efficacy in the treatment of tumor, chemotherapeutics may induce drug resistance during sequential treatment in almost all cancers. Chemoresistance to standard anticancer agents is a major problem and all clinically available drugs eventually fail [4]. The molecular or genetic basis of resistance to chemotherapy is

extraordinarily complex, involving multiple processes, such as drug transport and metabolism, DNA repair, and apoptosis regulation, etc. [5]. Therefore, total reversal of chemoresistance has not yet been achieved and remains area of hot pursuit. Despite significant improvements in new chemotherapeutic agents, more than 90% of cancer patients ultimately die because currently there are no chemotherapeutic options available that are completely unaffected by chemoresistance.

Paclitaxel is a natural compound originally isolated from pacific yew tree bark and now is used to treat patients with a variety of cancers, including lung, ovarian, breast cancer, head and neck cancer [6], gastric cancer [7], and colon cancer [8], etc. The anticancer mechanism of paclitaxel is mediated by arresting microtubular polymerization and inducing apoptosis in cancer cells through binding to and inhibiting an apoptosis-stopping protein called Bcl-2 [9, 10]. Paclitaxel is a classic chemotherapeutic drug that induces multidrug resistance (MDR) in many of the treated cancers, including colorectal cancer [11, 12]. Developing agents to reverse chemoresistance could sensitize the anticancer effect of currently available chemotherapeutic agents, which would improve patients' survival.

MicroRNAs (miRNA) are a class of endogenous short (19–24 nucleotides) noncoding RNAs, which function primarily to down regulate gene expression by specifically binding to the 3′-untranslational region (3′-UTR) of mRNAs. The binding of miRNA can prevent mRNA translation and/or promote degradation [13, 14]. Notably, a single miRNA can regulate thousands of genes, while a single gene may be regulated by more than one miRNA [15]. Recent evidence demonstrated that miRNAs function as oncogenes or tumor suppressors to modulate multiple oncogenic cellular processes, including cell proliferation, apoptosis, invasion, and metastasis [16]. MicroRNA 22 (miR-22) is a 22-nt non-coding RNA and was originally identified in HeLa cells as a tumor-suppressing miRNA. Subsequently, miR-22 was identified to be ubiquitously expressed in a variety of tissues [16]. Recently, several targets of miR-22 were reported to mediate its tumor-suppressive effect. For example, miR-22 was revealed to upregulate tumor-suppressive PTEN and Max genes and downregulate oncogene c-myc expression, etc. [15–17]. However, the downstream signaling of the miR-22-regulated targets and their involvements in chemoresistance of colon cancer has not yet been clarified.

In this study, the roles and mechanisms of miR-22 in reversing chemoresistance in *p53*-mutated and *p53* wild-type colon cancer cells were examined. A significant sensitizing role of miR-22 on the cytotoxicity of paclitaxel was observed in the *p53*-mutated colon cancer cells and the signaling pathway that mediated the suppressive role of miR-22 was elucidated. Our study highlighted the role of miR-22 in chemoresistance and its possible application in colon cancer therapy in the clinic.

Materials and methods

Cell culture

HT-29 and HCT-15 cells are human colorectal adenocarcinoma cells, expressing mutated p53. HCT-116 is a human colon carcinoma cell line, which contains wild-type p53 gene. These three cell lines were acquired originally from American Type Culture Collection (ATCC) and cultured in DMEM medium (Invitrogen Inc., Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml of streptomycin at 37°C, 5% CO_2.

Construction of miR-22 expression vector

The miR-22 expression vector was constructed according to a previously published protocol (18). Briefly, a 22-nt human miR-22 minigene sequence (5′-AAGCTGCCAG TTGAAGAACTGT-3′) was synthesized for transcription of miR-22. Two complementary oligonucleotides were annealed to yield the 22-nt miR-22 expression minigene. A tract of six thymidines was designed at the 3′ end to terminate RNA pol III transcription. BamHI- and HindIII-compatible overhangs were introduced at each end to facilitate ligation into the pSilencer-3.0 vector (Ambion, Inc., Austin, TX, USA). An H1 promoter is upstream of the BamHI site to initiate transcription precisely at the first nucleotide of the miR-22. As a negative control, a 22-nt sequence with no known target in the human genome was cloned into the same vector in place of the miR-22 minigene.

Northern blot analysis

Total RNA was extracted from control or miR-22 expressing vector-transfected cells using Trizol reagent (Invitrogen). The micoRNA was further isolated with PureLink™ miRNA isolation kit (Invitrogen). A total 2 μg of small RNA was loaded onto a 2.5% denaturing formaldehyde gel and transferred to a nylon membrane. Prehybridization and hybridization were performed with the ExpressHyb buffer (Invitrogen) at 68°C. The membrane was rinsed following the manufacturer's instructions (Invitrogen) and developed for signal detection. To detect miR-22 RNA, the antisense oligonucleotide of miR-22 (5′-ACAGTTCTTCAACTGGCAGCTTC-3′) was ³²P-labeled by using [γ-³²P]ATP and T4 polynucleotide kinase. The ³²P-end labeled oligonucleotide probe was used directly for hybridization. The band size of miR-22 was determined by

the ZR small-RNA[TM] ladder (ZYMO Research, Orange, CA, USA).

MTT assay for cell proliferation

The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay for cell proliferation in miR-22-transfected and/or paclitaxel-treated cells was carried out using an established protocol [19]. The MTT assay examines the activity of metabolic enzymes in the mitochondria of live cells. Therefore, MTT can reflect the viability and amount of cells. Cells that were grown to 80–90% confluency in 96-well plate were transfected with control vector or miR-22 expressing vector using lipofectamine 2000 (Invitrogen). Twenty-four hours later, cells were treated with 0, 0.5, 5, 25, and 50 nM of paclitaxel (Bedford Laboratories, Bedford, OH, USA) for 24 h. The cells were then subjected to MTT assay [19]. The OD values were normalized to the cells that were transfected with control vector, but without treatment of paclitaxel.

Hoechst staining for apoptotic cells

Cellular apoptosis induced by miR-22 transfection and/or paclitaxel was observed by Hoechst33342 (Calbiochem, San Diego, CA) staining. A published method for Hoechst staining was adopted [19]. This method allows for the observation of nuclear morphology. Cells at 50–60% confluency were transfected with control vector or miR-22 expressing vector using lipofectamine 2000 (Invitrogen). Twenty-four hours later, cells were treated with 0, 0.5, 5, 25, and 50 nM of paclitaxel for 24 h. Cells were fixed in methanol/acetic acid (3:1) for 10 min at 4°C, followed by staining with Hoechst33342 (5 μg/ml) for 10 min at room temperature. Apoptosis was evaluated under a fluorescence microscope. Cells that showed clear condensation and small bright nuclei were counted as apoptotic cells. To get a quantitative value, five randomly chosen areas were counted and averaged for each sample.

Clonogenic assay

Cells at 50–60% confluence were transfected with control vector or miR-22 expressing vector using lipofectamine 2000. Twenty-four hours later, cells were treated with 0, 0.5, 5, 25, and 50 nM of paclitaxel for 24 h. They were then plated in 10 cm dishes at a density such that 100 colonies could grow in the control vector-transfected cells. Ten days later, cells on the dishes were fixed in methanol and stained with 2% Giemsa solution. Colonies with over 50 cells were counted. Five replicate dishes were plated for each dose. The colonies were counted.

Tissue homogenate and Western blot

Cells were homogenized and Western blot was performed as previously described [18]. The antibodies for PTEN, phospho-PTEN, phospho-Akt Ser[473], total Akt, site-specific phosphorylation of the PI3 K p85 subunit, p53, Bax, active caspase-3, and the peroxidase-labeled secondary antibody were purchased from Cell Signaling Technology (Beverley, MA, USA). The anti-MTDH antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). To control for loading efficiency, the blots were stripped and reprobed with α-tubulin antibody (Sigma-Aldrich).

Results

Cell proliferation assay

Figure 1 shows the results of MTT assay. The MTT assay allows quantification of live cells [19]. HT-29 cell is a *p53*-mutated cell. Paclitaxel (0.5, 5, 25, and 50 nM) decreased cell viability (7, 18, 23, and 27%). Transfection of miR-22 vector resulted in 29% of decrease in the HT-29 viability and significantly sensitized the inhibitory role of paclitaxel at all of the tested concentrations (36, 47, 49, and 54% decreases for 0.5, 5, 25, and 50 nM of paclitaxel, respectively) (Fig. 1a). Compared with the HT-29 cell, HCT-15 cell (*p53*-mutated) is a little more sensitive to paclitaxel and 0.5, 5, 25, and 50 nM of paclitaxel induced 13, 39, 53, and 58% of decrease in cell viability, respectively. Transfection of miR-22 resulted in 30% of decrease in cell viability and significantly sensitized the inhibitory role of paclitaxel (35, 55, 66, and 67% decreases for 0.5, 5, 25, and 50 nM of paclitaxel, respectively) (Fig. 1b). In contrast to both HT-29 and HCT-15 cells, HCT-116 cell (*p53* wild type) is more sensitive to paclitaxel and 0.5, 5, 25, and 50 nM paclitaxel induced 52, 54, 67, and 64% of decrease in cell viability. Notably, transfection of miR-22 played no effect either in reducing cell viability or sensitizing the inhibitory role of paclitaxel (Fig. 1c).

Cell apoptosis assay

Figure 2 shows the results of Hoechst33342 staining, which was used to quantify apoptosis. The Hoechst33342 dye stains the nuclei of mammalian cells. Apoptotic cells are typically identified as cells that possess significantly condensed and smaller nuclei. In the HT-29 cell, 5, 25, 50 nM of paclitaxel only induced about 9, 7, and 8% apoptosis, respectively. Conversely, transfection of miR-22 induced 30% apoptosis in the HT-29 cells and significantly sensitized the apoptotic role of paclitaxel at all of the tested
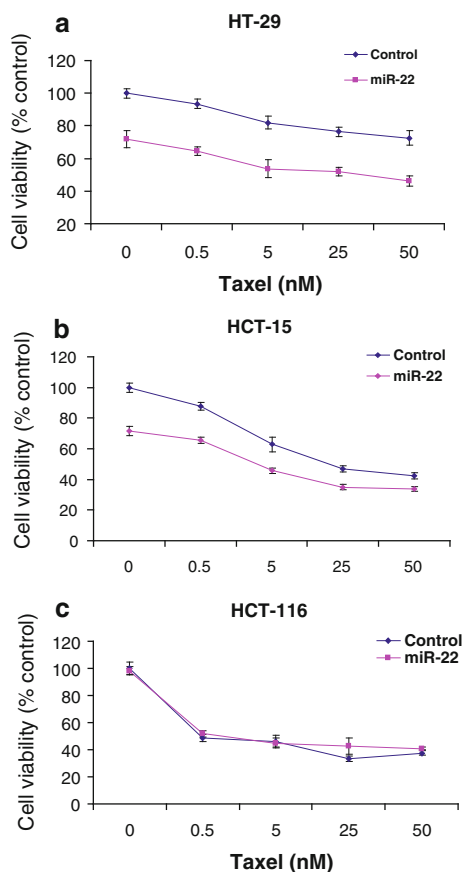
Fig. 1 MTT assay of cell viability. Cells were treated as described in the Methods section. The OD value was normalized with that of the control vector-transfected cells, but without paclitaxel treatment. **a** MTT assay of HT-29 cells. HT-29 cell was resistant to paclitaxel. Transfection of miR-22 expressing vector significantly decreased cell viability and sensitized the inhibitory role of paclitaxel. $P < 0.0001$, $n = 10$. **b** MTT assay of HCT-15 cells. HCT-15 cells were relatively resistant to paclitaxel and transfection of miR-22 vector decreased cell viability and sensitized the inhibitory role of paclitaxel. $P < 0.0001$, $n = 10$. **c** MTT assay of HCT-116 cells. HCT-116 cells were sensitive to paclitaxel and transfection of miR-22 expressing vector played no effect in cell viability and the inhibitory role of paclitaxel on cell viability. $P > 0.05$, $n = 10$

concentrations (37, 39, and 38% apoptosis for 5, 25, and 50 nM of paclitaxel, respectively) (Fig. 2b). In the HCT-15 cells, 5, 25, and 50 nM of paclitaxel induced 31, 49, and 50% apoptosis, respectively. Transfection of miR-22 induced 26% apoptosis and significantly sensitized the apoptotic role of paclitaxel (50, 52, and 56% apoptosis for 5, 25, and 50 nM of paclitaxel, respectively) (Fig. 2d). In contrast to HT-29 and HCT-15 cells, HCT-116 cells are more sensitive to paclitaxel and 5, 25, and 50 nM concentration of paclitaxel induced 36, 36, and 43% apoptosis, respectively. Notably, transfection of miR-22 played no effect either in inducing apoptosis or sensitizing the apoptotic role of paclitaxel (Fig. 2f).

Cell survival assay

Figure 3 shows the results of a clonogenic assay, which evaluates the long-term cellular survival after treatments. In HT-29 cells, compared to cells that have been transfected with control vector, the transfection of miR-22 significantly decreased the survival and enhanced the inhibitory role of paclitaxel. Treatment with 0.5, 5, 25, and 50 nM of paclitaxel induced 8, 44, 63, and 68% of decrease in cellular survival in control vector-transfected cells, while induced 60, 86, 90, and 95% of decrease in survival in miR-22-transfected cells (Fig. 3a). In HCT-15 cells, transfection of miR-22 significantly decreased the survival (38% in decrease) and enhanced the inhibitory role of paclitaxel. Treatment with 0.5, 5, 25, and 50 nM of paclitaxel induced 11, 48, 75, and 85% of decrease in cellular survival in control vector-transfected cells, but induced 50, 71, 91, and 97% of decrease in survival in miR-22-transfected cells (Fig. 3b). In contrast to its effect in HT-29 and HCT-15 cells, paclitaxel induced a significant decrease in cellular survival in HCT-116 cells at 0.5, 5, 25, and 50 nM of paclitaxel, which induced 51, 75, 91, and 97% of decrease in cellular survival, respectively. Notably, transfection of miR-22 played no effect either in reducing survival or sensitizing the inhibitory role of paclitaxel (Fig. 3c).

Signaling transduction assay

Figure 4a shows the results of Northern blot assay. Expression of miR-22 was detected at a very low level in the HT-29 and HCT-15 cells, but at a relatively high level in the HCT-116 cells. Transfection of miR-22 vector-induced overexpression of miR-22. Figure 4b shows the results of Western blot assay. In the HCT-116 cells, transfection of miR-22 vector exhibited no effect on the expression of all tested molecules. In both the HT-29 and HCT-15 cells, transfection of miR-22 vector increased PTEN and phospho-PTEN levels, decreased Akt Ser[473] and MTDH levels, and increased Bax and active caspase-3 levels. But, in contrast to HT-29 cell, transfection of miR-22 vector decreased p53 expression in HCT-15 cells (Fig. 4b).

Discussion

Chemotherapy, the first-line of treatment for a large number of patients with unresectable colorectal cancer, provides a modest improvement to overall survival. However, one of the major problems in treating colon cancer is the development of chemoresistance to cytotoxic chemotherapeutic agents [20]. Paclitaxel has been widely used to treat
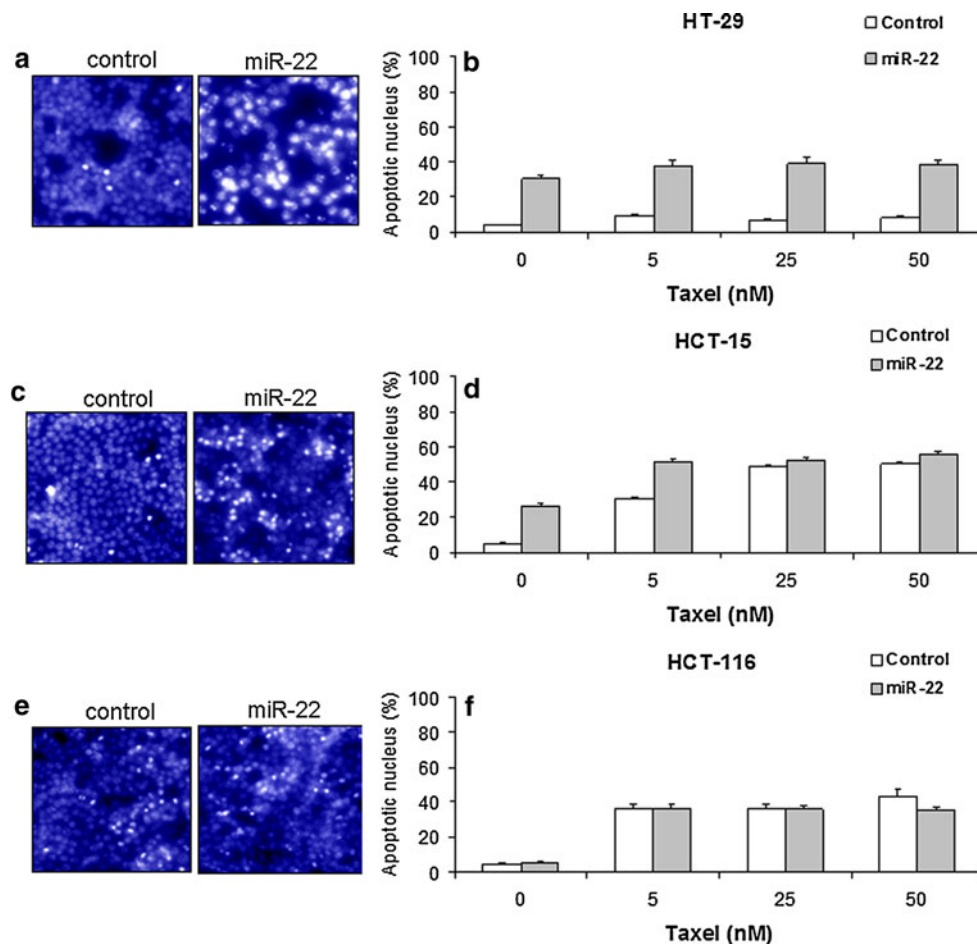
Fig. 2 Hoechst33342 staining of apoptosis. Cells were treated as described in the Methods section. **a** Hoechst33342 staining of HT-29 cells transfected with control vector and vector expressing miR-22, without treatment with paclitaxel. Transfection of miR-22 vector obviously increased apoptotic nuclei. **b** Calculation of apoptotic nuclei in paclitaxel-treated HT-29 cells. Transfection of miR-22 expressing vector significantly sensitized the apoptotic role of paclitaxel. **c** Hoechst33342 staining of HCT-15 cells. Transfection of miR-22 expressing vector obviously increased apoptotic nuclei. **d** Calculation of apoptotic nuclei in paclitaxel-treated HCT-15 cells. Transfection of miR-22 expressing vector significantly sensitized the apoptotic role of paclitaxel. **e** Hoechst33342 staining of HCT-116 cells. Transfection of miR-22 vector played no effect on cell apoptosis. **f** Calculation of apoptotic nucleus in paclitaxel-treated HCT-116 cells. Transfection of miR-22 expressing vector did not sensitize the apoptotic role of paclitaxel. $P < 0.0001$, $n = 5$ for HT-29 and HCT-15 cells. $P > 0.05$, $n = 5$ for HCT-116 cells

a variety of cancers, including colon cancer; however, it is also known to induce MDR. In this study, we demonstrated that overexpression of miR-22 enhanced the anticancer effect of paclitaxel in the *p53*-mutated cells through increasing cell apoptosis and reducing cell proliferation and survival. The anticancer role of miR-22 was mediated by activation of PTEN signaling, subsequent inhibition of Akt Ser[473] phosphorylation and MTDH expression, as well as upregulation of Bax and active caspase-3 levels. The chemosensitive role of miR-22 in the *p53*-mutated cells highlighted the application of miR-22 as a sensitizer of chemotherapeutic drugs of colon cancer.

Resistance to anticancer agents is one of the primary obstacles for effective cancer therapy, but the underlying mechanisms remain controversial [11]. The phosphatidylinositol 3-kinase

(PI3K) pathway is known to be involved in the regulation of cell survival, apoptosis and growth. The accumulated evidence demonstrated that activation of Akt via PI3K is involved in chemoresistance [21–23]. PI3K produces IP3, which activates Akt through phosphorylation of Akt at serine 473 and threonine 308 residues [24]. Activated Akt can inhibit apoptosis and increase cell survival by negatively regulating Bax levels [25]. PI3 kinase is a key signaling molecule negatively regulated by PTEN. PTEN antagonizes PI3K-Akt signaling through removing the phosphate from PI3K-produced IP3 to generate IP2 [26]. However, previous studies demonstrated that p53 can negatively regulate PTEN activation [27] and miR-22 was identified as a tumor-suppressive miRNA to upregulate PTEN expression [15, 16]. Whether miR-22 exhibits different biological behaviors in the
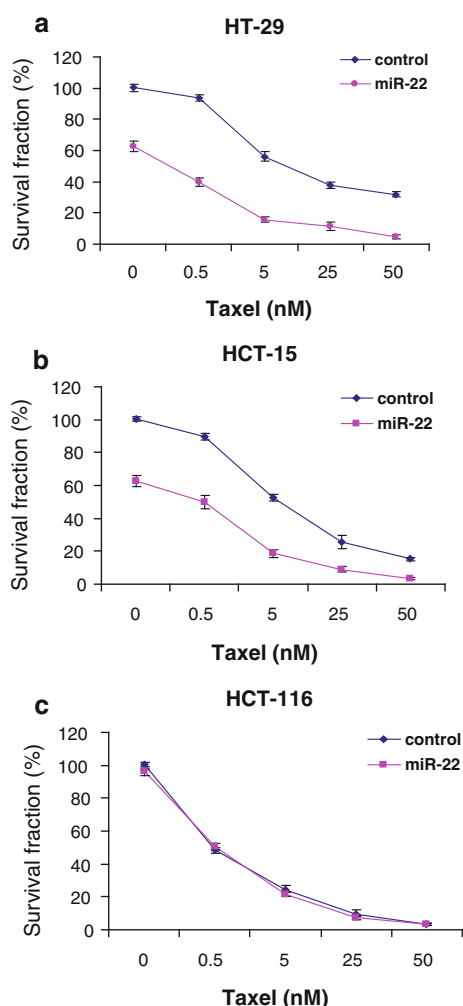
Fig. 3 Clonogenic assay of cellular survival. Cells were treated as described in the Methods section. Survival colonies were calculated and plotted with paclitaxel treatment. **a** Cellular survival in the HT-29 cells. **b** Cellular survival in the HCT-15 cells. **c** Cellular survival in the HCT-116 cells. Transfection of miR-22 expressing vector significantly decreased cellular survival and sensitized the role of paclitaxel in the HT-29 and HCT-15 cells ($P < 0.0001, n = 5$), but played no effect in the HCT-116 cells ($P > 0.05, n = 5$)

Fig. 4 miR-22 expression and the signaling. **a** Northern blot of miR-22 expression. Very low miR-22 levels were detected in the HT-29 and HCT-15 cells. A relatively higher miR-22 expression was seen in the HCT-116 cells. Cells were transfected with miR-expression vector for 24 h. Transfection of miR-22 vector showed a high expression of miR-22. **b** Western blot assay of protein or phosphorylation levels in control vector (C) and miR-22 vector (R) transfected HT-29, HCT-15, and HCT-116 cells. Cells were transfected with miR-expression vector or control vector for 24 h

p53-mutated and p53 wild-type colon cancer cells has not been addressed previously. In this study, we found that miR-22 overexpression upregulated PTEN protein and phosphorylation levels only in the *p53*-mutated HT-29 and HCT-15 cells. Overexpression of miR-22 in p53 wild-type HCT-116 cells did not affect PTEN expression, cell proliferation or apoptosis. How p53 antagonized the apoptotic role of miR-22 is currently unclear. Notably, overexpression of miR-22 reduced p53 protein level in HCT-15 cells. TargetScan (MIT, USA), an online software used to predict binding sites on mRNAs, predicted that the 3′-UTR of p53, PI3K, Akt, MTDH, and Bax genes do not incorporate a target site of miR-22. Therefore, it is currently unclear how p53 was regulated by miR-22.
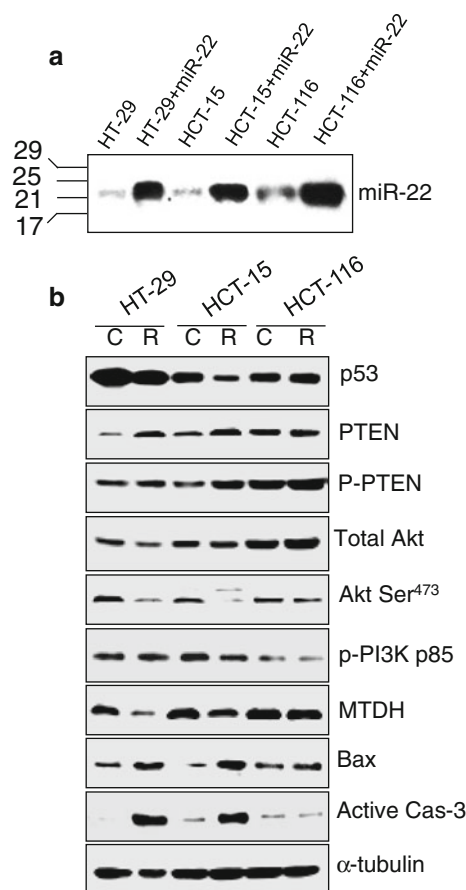
Metadherin (MTDH) has recently emerged as a crucial mediator of tumor malignancy and was reported to be involved in chemoresistance and metastasis [28, 29]. In vitro and in vivo chemoresistance analysis showed that MTDH knockdown sensitized several different breast cancer cell lines to paclitaxel, doxorubicin, cisplatin, and UV-radiation [19]. MTDH increased chemoresistance through promoting cell survival mediated by the prosurvival pathways, such as PI3K and NFκB [30, 31]. MTDH acts both as a downstream target of Akt and as an upstream activator of the PI3K-Akt pathway, though the mechanism of how PI3K signaling is activated by MTDH is still unclear [32]. We demonstrated that miR-22-induced upregulation of PTEN expression and subsequent downregulation of Akt activation was accompanied by the decrease in MTDH expression. Importantly, PI3K activation revealed by p85 subunit phosphorylation showed no

changes in both *p53*-mutated and *p53* wild-type colon cancer cells. This suggested that MTDH is a downstream molecule of activated Akt and other downstream genes of MTDH may directly regulate chemoresistance.

In the clinic, *p53* mutations were found in 34% of the proximal colon tumors and in 45% of the distal colon and rectal tumors [33]. Notably, colorectal cancer patients with wild-type *p53* exhibited significantly better survival when treated with chemotherapy compared to patients with mutated *p53* [33]. We also found that *p53*-mutated colon cancer cells were relatively resistant to paclitaxel compared with the cells with wild-type *p53*. These findings suggest that p53 mutation is involved in the chemoresistance in colon cancer cells. Importantly, we found that miR-22 can reverse the chemoresistance in the *p53*-mutated colon cancer cells. This suggested that miR-22 is a promising sensitizer for the chemotherapeutic effect of drugs on colon cancer. However, delivery of miRNA as a treatment strategy has not yet been established in the clinic trials. The viral- or nonviral-based delivery, such as nano-particles-based delivery of miRNA can be developed to sensitize the cytotoxicity of chemotherapy agents, which would significantly benefit the patients' survival.

Our study demonstrated that *p53* mutation is associated with the chemoresistance of paclitaxel. Overexpression of miR-22 can reverse paclitaxel-induced chemoresistance through increasing cellular apoptosis and reducing cell proliferation and survival. The chemosensitive role of miR-22 was mediated by upregulating PTEN expression, subsequently downregulating Akt activation and MTDH expression, and elevating Bax and active caspase-3 levels. The chemosensitive role of miR-22 suggested that miR-22 can be developed as a novel sensitizer of chemotherapy. However, drug resistance was induced during sequential chemotherapeutic treatments. Therefore, it might be more relevant to test miR-22 in a pre-established tumor animal model that is not responsive to paclitaxel treatment.

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