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miR-195 Potentiates the Efficacy of Microtubule-Targeting Agents in Non-Small Cell Lung Cancer

Xiaojie Yu^{1,2}, Yiqiang Zhang¹, Xiuye Ma¹, and Alexander Pertsemlidis^{1,2,3,*}

¹Greehey Children's Cancer Research Institute, The University of Texas Health Science Center at San Antonio, TX 78229 USA

²Department of Cell Systems and Anatomy, The University of Texas Health Science Center at San Antonio, TX 78229 USA

³Department of Pediatrics, The University of Texas Health Science Center at San Antonio, TX 78229 USA

Abstract

Microtubule-targeting agents (MTAs) are widely used for the treatment of non-small cell lung cancer (NSCLC). The response rate is only ~25%, mainly attributable to drug resistance. To identify determinants of resistance in NSCLC, we performed a high-throughput screen using a library of miRNA mimics. Here we report that miR-195 synergizes with MTAs to inhibit the growth of NSCLC cells *in vitro*, that increased expression of miR-195 sensitizes NSCLC cells to MTAs and that repression of miR-195 confers resistance to MTAs. We show that NSCLC tumors over-expressing miR-195 are more sensitive to MTA treatment and that induced expression of miR-195 in NSCLC tumors potentiates the anti-tumor effect of MTAs. Additionally, we demonstrate that miR-195 targets checkpoint kinase 1 (CHEK1) to regulate the response of NSCLC cells to MTAs, that over-expression of CHEK1 contributes to resistance to MTAs and that knock-down of CHEK1 synergizes with MTAs to repress cell growth. Our results highlight the importance of miR-195 in regulating the response of NSCLC cells to MTAs and underline the potential application of miR-195 as a biomarker for response to MTAs, and as a therapeutic adjuvant to MTA treatment.

Keywords

miR-195; CHEK1; non-small cell lung cancer; paclitaxel; eribulin

*To whom correspondence should be addressed. Tel: (210) 562-9062; Fax: (210) 562-9014; pertsemlidis@uthscsa.edu.

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Declarations of interest: none

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

1. INTRODUCTION

Lung cancer remains the deadliest cancer globally, accounting for about 25% of all cancer deaths [1]. Non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma and large cell carcinoma, constitutes over 85% of lung cancer. Microtubule-targeting agents (MTAs) are widely used for the treatment of NSCLC. MTAs are a family of chemotherapeutic agents that bind different sites on microtubules and disrupt their structures and dynamics, thereby arresting cell cycle progression at mitosis and leading to cell death [2, 3]. The response rate to MTAs in NSCLC is only 20–30% [4–6]. To overcome this hurdle and improve outcomes, we need either a biomarker to predict patient response and enable stratification prior to treatment, or an adjuvant strategy by which efficacy can be increased.

microRNAs (miRNAs) are a family of small (18–24 nt) non-coding RNAs that post-transcriptionally repress gene expression by interacting with the 3' untranslated regions (UTRs) of their targets [7]. miRNAs have been shown to regulate nearly all aspects of cancer, including the canonical hallmarks [7–11]. miRNAs that are down-regulated or over-expressed in cancer are potential candidates for therapeutic intervention. In addition, miRNAs that regulate the response of cancer cells to chemotherapy can be over-expressed or inhibited as adjuvants to cancer treatment. As one example, anti-miR-155 liposomal nanoparticles, designed to repress miR-155 to overcome miR-155-induced resistance to chemotherapy, have recently been shown to re-sensitize lung tumors to chemotherapy [12].

miR-195 is located at chromosome 17p13, one of the most frequently deleted regions in cancer. miR-195 has been shown to be down-regulated in NSCLC and to repress the growth and progression of NSCLC cells [13–17]. We have demonstrated that miR-195 regulates the apoptosis and senescence of NSCLC cells [18]. In addition, miR-195 has been shown to enhance the radiosensitivity of breast cancer and colorectal cancer through its regulation of *BCL2* and *CARM1*, respectively [19, 20]. miR-195 has also been shown to sensitize breast cancer cells to doxorubicin by targeting *RAFI*, and hepatocellular carcinoma to fluorouracil by targeting *BCL2L2* [21, 22]. In contrast, miR-195 has also been reported to correlate with the resistance of cervical cancer cells to temozolomide [23]. These reports reveal a diversity of roles for miR-195 in regulating the response of cancer cells to therapeutic agents. However, the demonstrations were limited to *in vitro* cell culture; no *in vivo* results have been published to support the role of miR-195 in modulating the response of tumors to treatment. Additionally, it is not clear whether miR-195 regulates the response of NSCLC to MTAs, and if so, how the response is mediated.

In this study, we performed a high-throughput screen and identified synergy between miR-195 and MTAs in inhibiting the growth of NSCLC cells. We found that over-expression of miR-195 sensitizes NSCLC to MTAs both *in vitro* and *in vivo* and that induced expression of miR-195 potentiates the efficacy of eribulin to repress lung tumor growth. Additionally, we demonstrated that knockout of miR-195 confers resistance to MTAs in NSCLC cells. We established that miR-195 targets *CHEK1* to regulate the response of NSCLC cells to MTAs. We demonstrated that the ratio of miR-195 expression to *CHEK1* expression in tumors is significantly associated with both recurrence-free and overall survival of lung adenocarcinoma patients.

2. MATERIALS AND METHODS

2.1. Reagents and Cell Lines

Paclitaxel was obtained from Teva Pharmaceutical Industries (PA, USA). Eribulin mesylate (Eisai, Inc.) was kindly provided by Dr. Peter Houghton. miR-195 mimics were purchased from Dharmacon (CO, USA) and IDT (IA, USA). Two negative control oligos were purchased from Dharmacon (D-001810-10-05 and CN-001000-01-20). Two miR-195 inhibitors, IH-300643-05-0005 (miR-195 inh #1) and HSTUD0320 (miR-195 inh #2), were obtained from Dharmacon and Sigma-Aldrich (MO, USA). Lipofectamine RNAiMAX and 2000 transfection reagents were purchased from Thermo Fisher (MA, USA). Oligos were transfected into cells at 25 nM unless otherwise specified.

Two siRNAs designed against *CHEK1* were purchased from Sigma-Aldrich:

SASI_Hs02_00326304 targeting CTGAAAGAGACTTGTGAGAA, and

SASI_Hs02_00326305 targeting TAGATATGAAGCGTGCCGT.

NSCLC cell lines were established at the National Cancer Institute and obtained from Dr. John Minna at the Hamon Center for Therapeutic Oncology Research at UT Southwestern Medical Center in Dallas, Texas. All cell lines were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were grown in a humidified atmosphere with 5% CO₂ at 37°C, authenticated using short tandem repeat profiling, and confirmed to be mycoplasma-free through PCR. Cells were discarded when they were close to passage 20.

H1299/Control and H1299/miR-195 cells were generated from luciferase-pcDNA3, which was a gift from William Kaelin (Addgene plasmid # 18964). Two H1299 colonies of doxycycline-inducible ptet-miR-195 cells were generated using the Mir-X™ Inducible miRNA System (Clontech) following manufacturer instructions. The two colonies were named H1299/ptet-miR-195 #3 and #7. Briefly, we first generated and screened H1299/ptet cells using G418. Then we used H1299/ptet cells to further generate miR-195-inducible H1299/ptet-miR-195 cells screened by puromycin. *MIR195* was knocked out using pSpCas9(BB)-2A-GFP (PX458) (Addgene plasmid # 48138) and pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid # 62988), which were gifts from Feng Zhang, following reported protocols [24]. Two sgRNAs were cloned into PX458 and PX459 separately and co-transfected into H1299 cells, which were further treated with puromycin (1 µg/mL) for 3 days. Single colonies were picked and validated by PCR. Candidate cell colonies validated by PCR were further confirmed by sequencing through GenScript (NJ, USA).

Sequences for sgRNA1 were:

sgRNA1-forward: CACCGGGTGGTGAAAACCTACCGAGG, and

sgRNA1-reverse: AAACCCTCGGTAGTTTTTCACCACCC.

Sequences for sgRNA2 were:

sgRNA2-forward: CACCGTTGAGGCAGAACTTACTCCC, and

sgRNA2-reverse: AAACGGGAGTAAGTTCTGCCTCAAC.

Two pairs of primers for validation were purchased from Sigma-Aldrich:

Primer 1-forward: GCTATGTGCTCTCTTCCTTTC, and

Primer 1-reverse: TTCGTGCTGTCTGCTTAAC.

Primer 2-forward: TCTTCCCAGCACTGCTAT, and

Primer 2-reverse: CTGTTCCCTCTTCTCTCCTC.

2.2. High-Throughput Screen

The screen was designed with two arms, one assessing the effect of miRNA mimics on cell viability and the other assessing the degree to which miRNA mimics sensitize NSCLC cells to paclitaxel. A library containing 1,239 miRNA mimics was obtained from GE Dharmacon (CS-001010 Human Mimics Lot 09167 and CS-001015 Supplement Human Mimic 16.0 Lot 11144). The library was arrayed in a one-mimic/one-well format in the central 60 wells of 96-well micro-titer plates. Reverse transfections of mimics (25 nM) into NSCLC cells were performed in triplicate. 24–48 h after transfection, cells were treated with carrier (medium) or 10 nM paclitaxel. After total incubation for 120 h, cell viability was assessed by CellTiter-Glo cell viability assay (Promega). Each miRNA mimic was assigned a relative viability calculated by normalizing replicate means to the mean of the central 60 wells on each plate to assess the effect of each mimic on cell viability in the absence (V_{carrier}) or presence of paclitaxel ($V_{\text{paclitaxel}}$). Each miRNA mimic was also assigned a synergy value calculated as mean viability in the presence of paclitaxel divided by mean viability in the presence of carrier ($\text{Synergy} = V_{\text{paclitaxel}}/V_{\text{carrier}}$) to assess the effect of each miRNA mimic on cellular response to paclitaxel.

2.3. Cell Viability Assays

Cells were plated in 96-well format and/or transfected with oligos in different concentrations. After incubating for 24–48 h, cells were further treated with medium or different concentrations of drugs. After total incubation for 120 h, cell viability was determined using the CellTiter-Glo® cell viability assay (Promega). Dose-response curves were generated using GraphPad Prism 7 (CA, USA).

2.4. RNA Extraction and qRT-PCR

Total RNA was prepared using the mirVana miRNA Isolation Kit (Ambion) according to manufacturer instructions. Intracellular miRNA levels were assessed by qRT-PCR on an ABI ViiA7 System using miRNA expression assay primer and probe sets (Applied Biosystems). RNU44, RNU66 and U6 were used as controls for normalization of miRNA expression. mRNA levels were assessed by qRT-PCR on an ABI ViiA7 System using SYBR Green (Thermo Fisher) and primers designed by IDT and purchased from Sigma. *GAPDH* expression was used as a control for normalization of mRNA expression. Threshold cycle times (Ct) were obtained, and relative gene expression was calculated using the comparative cycle time method.

Primers used for *GAPDH*:

forward-GAAGGTGAAGGTCGGAGTC, and

reverse-GAAGATGGTGATGGGATTTC.

Primers for *CHEK1*:

forward-TTGTCTCCACCTCATCATAAC, and

reverse-ATGCCTATGTCTGGCTCTATTC.

2.5. mRNA Expression Profiling

H358 and H1993 cells were transfected with miR-195 mimic (25 nM) or mock transfected. 24 h after transfection, total RNA was prepared as described above. At least a several-hundred-fold increase of intracellular levels of miR-195 was detected in cells transfected with miR-195 by qRT-PCR, confirming that the transfections were successful. Gene expression profiling was performed using the HumanHT-12 v4 Expression BeadChip through the Genomics and Microarray Core Facility at UT Southwestern Medical Center.

2.6. Luciferase Reporter Assay

Luciferase reporter assays were performed as previously reported [25]. Mutant constructs were generated with the seed target sites mutated or deleted as specified above under **Results**. H1299 cells were maintained in 96-well plates and co-transfected with luciferase reporters (0.05 µg/well) and miRNA mimics or control oligo (15 nM). Luciferase activities were measured 72 h later using the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase was normalized to sea pansy luciferase activity to evaluate the regulatory effect of miR-195 on its putative targets.

Primers for *CHEK1* 3'UTR:

forward-GACGTGAGAGCTCGGACCATCGGCTCTGGGGAAT, and

reverse-GTAGTAGCTCGAGCTGGAAACTCATGTCCCCTGAAACTTGG.

Primers for *CHEK1* 3'UTR with deletion of the binding site of miR-195:

forward-

TAGCTGAGAGCTCTATGTTGACATTATTCTTCCTAGAGAAGATTATCCTGTCC
TGCA, and

reverse-GTAGTAGCTCGAGCTGGAAACTCATGTCCCCTGAAACTTGG.

2.7. Western Blots

Cell lysates were prepared using RIPA buffer. Equal amounts of lysate were resolved by SDS-PAGE and transferred to ImmunBlot PVDF membranes (Bio-Rad). Membranes were blocked and probed with specific primary antibodies. Antibodies to Chk1 (A300-161A-T) and pChk1 (A304-673A-T) were purchased from Bethyl Laboratories (TX, USA). Antibodies to calnexin (sc-11397) and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology (TX, USA). Actin antibody (4970P) was purchased from Cell Signaling Technology (MA, USA). Bound antibodies were detected with secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology) and visualized

by enhanced chemiluminescent (ECL) substrate (Pierce/Thermo Fisher) on an Odyssey Fc Imaging System (LI-COR). All western blots were repeated at least twice.

2.8. Synergy Evaluation

Bliss independence and Loewe additivity models were used to evaluate synergy. The Bliss independence model evaluates drug-drug interactions by comparing the effect of combining two drugs with their theoretical additivity [26–28]. Predicted additivity was calculated at each dose matrix point based on the single agent response curves. For a combination point at concentrations X , Y , the corresponding single agent effect levels I_X and I_Y were determined and the threshold value was calculated as $I_{Predicted} = I_X + I_Y - I_X I_Y$. The Loewe additivity model evaluates drug-drug interactions in terms of the combination index (CI), which indicates synergy if $CI < 1$, additivity if $CI = 1$, or antagonism if $CI > 1$ [29, 30]. CI values were calculated using CompuSyn [31].

2.9. Animal Experiments

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. Six- to seven-week-old female athymic nude Foxn1nu (nu/nu) mice were purchased from Envigo. 1×10^6 cells in 200 μ l PBS/Matrigel (v/v, 1:1) were injected subcutaneously into the flanks of the mice. Tumor volumes were measured using calipers and calculated by the formula: volume = (width)² × length/2.

Control cells (H1299/Control) and miR-195-overexpressing cells (H1299/miR-195) were used to assess the response of xenografts to eribulin. Mice were separated into four groups, with first two groups injected with H1299/Control cells and the other two groups injected with H1299/miR-195 cells. When tumors reached around 100 mm³, the first and third groups were IV injected with saline and the second and fourth group were IV injected with 0.5 mg/kg eribulin. Saline and eribulin were injected on Monday, Wednesday and Friday for two weeks.

We also assessed the response of xenografts to eribulin using miR-195-inducible cells. Mice were injected with #3 cells in the left flank and #7 cells in the right flank and separated into four groups. When tumors reached around 100 mm³, the first group was fed with water containing 2% sucrose and intravenously (IV) injected with vehicle (ethanol:water, 5:95), the second group was fed with water containing 2% sucrose plus 2 mg/mL doxycycline and IV injected with vehicle, the third group was fed with water containing 2% sucrose and IV injected with 0.5 mg/kg eribulin, and the fourth group was fed with water containing 2% sucrose plus 2 mg/mL doxycycline and IV injected with 0.5 mg/kg eribulin. Water was replaced every two days. Eribulin was injected four times at four day intervals and an additional two times after a one-week recovery.

2.10. Statistical Analysis

Student's t-test was used to compare mean values between different groups and two-way ANOVA was used to compare tumor growth curves, both as implemented in GraphPad

Prism 7, with $p < 0.05$ considered statistically significant. Results are representative of 2 to 4 individual experiments or as indicated in the figures.

3. RESULTS

3.1. A high-throughput screen (HTS) identified miR-195 as synergizing with MTAs in NSCLC

The founding member of the class of microtubule-targeting agents, paclitaxel, was isolated over 50 years ago and has been used to treat ovarian, breast and non-small cell lung cancers for decades. In order to identify miRNAs that regulate both the growth of NSCLC cells and their response to paclitaxel, we screened a library of miRNA mimics in 3 NSCLC cell lines (H1155, H1993 and H358). We identified 44 miRNAs as significantly influencing both cell growth (based on average viability) and response to paclitaxel (based on average synergy) (Supplementary Table 1, Figure 1A,B). Subsequently, we analyzed miRNA expression in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) patients available through The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov>) and found that 13 of the 44 miRNAs were significantly changed in tumor tissues compared to adjacent normal tissues (Supplementary Table 2, Figure 1C). Collectively, we found that 1 miRNA (miR-195) represses the growth of NSCLC cells, sensitizes NSCLC cells to paclitaxel, and exhibits down-regulation in tumors relative to adjacent normal tissues in both LUAD and LUSC (Figure 1B,C).

Initially, we evaluated the synergy between miR-195 and paclitaxel, which we consider representative of traditional MTAs. Furthermore, we evaluated the synergy between miR-195 and eribulin, a recently discovered microtubule-destabilizing agent that disrupts microtubule dynamics [32]. We assessed the response of NSCLC cells to different concentrations of miR-195 mimic, MTAs, and the combination. Both Bliss independence and Loewe additivity models demonstrated that miR-195 synergizes with paclitaxel and eribulin to repress the growth of NSCLC cells (Figure 1D–I). The responses of H1299 and H1993 cells to paclitaxel and eribulin are not influenced by control oligo (Supplementary Figure 1). H358 cells, however, are slightly sensitized to paclitaxel and eribulin by control oligo.

Collectively, these data highlight the potential of miR-195 as an anti-cancer agent either alone or in combination with chemotherapy. Notably, in a pair of NSCLC cell lines derived from the same patient (H1993 and H2073, with the former sensitive to MTAs and the latter overexpressing MDR1 and drug resistant), we found both to be sensitive to miR-195 (Supplementary Figure 2B). Even in drug-resistant H2073 cells, miR-195 synergizes with paclitaxel to repress cell growth (Supplementary Figure 2C). These results suggest that miR-195 acts independently of the common mechanisms underlying drug resistance and hence will be a useful adjuvant therapy to chemotherapy.

3.2. miR-195 regulates the response of NSCLC to MTAs

To better evaluate the influence of miR-195 expression on the response of NSCLC cells to MTAs, we generated stable miR-195-overexpressing cells and found that over-expression of

miR-195 sensitizes NSCLC cells to paclitaxel and eribulin (Figure 2A–C). Additionally, we knocked out miR-195 using CRISPR/Cas9 with pair of sgRNAs designed against the *MIR195* locus. While we did not find any clones with complete loss of miR-195, we found three clones (KO #4, #5, #9) with around 50% down-regulation of miR-195 compared to unmodified or wildtype (WT) cells (Figure 2D). Both KO #4 and KO #5 are more resistant to paclitaxel and eribulin than WT cells (Figure 2E,F).

For *in vivo* demonstration of the influence of miR-195 on the response of lung tumors to MTAs, we used eribulin rather than paclitaxel, for reasons of solubility and toxicity – eribulin mesylate is water soluble, while paclitaxel is poorly soluble in water and is dissolved in polyethoxylated castor oil (Cremophor EL), which itself has been shown to be toxic [33]. We found that independent over-expression of miR-195 or delivery of a low dose of eribulin slightly repress tumor growth, but that the combination has a stronger inhibitory effect on tumor growth with no significant impact on body weight (Figure 3A–C). After evaluating miR-195 expression in tumor tissues, we found that miR-195 is only slightly higher in miR-195-overexpressing tumors than in control tumors (Figure 3B). However, we also found that two tumors had much higher miR-195 expression than the others: one tumor was in miR-195 group and the other was in the miR-195+eribulin group. We demonstrated that the tumor with higher miR-195 levels grew much slower than control tumors or other tumors in the miR-195 group, highlighting the potential of miR-195 to repress tumor growth. Additionally, we found that the tumor with much higher miR-195 in the miR-195+eribulin group regressed, underlining the ability of miR-195 to sensitize tumors to eribulin treatment (Figure 3A).

To mimic the clinical scenario in which anti-cancer agents are given after a tumor has formed, we generated miR-195-inducible H1299 cell lines, in which exposure to doxycycline induces co-expression of miR-195 with ZsGreen fluorescent protein. Doxycycline has no effect on miR-195 expression in control cells (H1299/pTet-CTL), but dramatically induces the expression of miR-195 and ZsGreen in two inducible clones (H1299/pTet-miR-195 #3 and H1299/pTet-miR-195 #7) (Figure 2G,I, Supplementary Figure 3A–C). As expected, the induced expression of miR-195 represses NSCLC cell growth and sensitizes cancer cells to paclitaxel and eribulin (Supplementary Figure 3B,D, Figure 2H,J).

In xenografts, we found that a low dose of eribulin inhibits tumor growth (Figure 3D,G). Doxycycline had no effect on tumor growth in xenografts based on H1299/pTet-miR-195 #3, but slightly repressed tumor growth in H1299/pTet-miR-195 #7. In both, the combination of doxycycline and eribulin strongly inhibited tumor growth. After evaluating miR-195 expression in tumor tissues, we found that doxycycline results in around a 2-fold induction of miR-195 (Figure 3E,H). This increase was sufficient to sensitize tumors to eribulin, suggesting that if miR-195 could be increased robustly, the combination with eribulin would dramatically repress tumor growth. Accordingly, in one mouse, miR-195 expression was induced by 13-fold and it led to nearly complete regression of tumor (Figure 3D,E). Finally, merging the data for the two inducible clones shows that induction of miR-195 or treatment with eribulin alone only slightly represses tumor growth, while the combination of the two results strong inhibition of tumor growth (Supplementary Figure 4). However, none of the treatments have significant effects on mice body weight (Figure 3F,I).

3.3. miR-195 targets *CHEK1* to regulate the response of NSCLC cells to MTAs

To identify the gene that mediates the function of miR-195 in NSCLC, we profiled two NSCLC cell lines (H358 and H1993) for mRNA expression following transient transfection of miR-195 mimic. As previously reported, we examined cancer-related genes harboring predicted target sites for miR-195 (“TGCTGCT” sequences) in their 3’UTRs, whose expression decreased by more than 2-fold (*manuscript accepted to Cell Death & Disease*). Among the top candidate targets, *CHEK1* has been shown to be a direct target of miR-195 in NSCLC [14]. Intriguingly, *CHEK1* has also been shown to regulate the response of various cancers to chemotherapy, including MTAs. Chemical inhibitors for *CHEK1* have been developed and are currently being evaluated in clinical trials [34–38]. Based on these observations, we anticipated that *CHEK1* is a direct target of miR-195 and mediates response of NSCLC cells to MTAs.

We used qRT-PCR and western blot to confirm the inhibition of *CHEK1* by miR-195 in NSCLC cells (Supplementary Figure 5A–E). One binding site of miR-195 was found in the 3’UTR of *CHEK1*. We validated the direct and specific binding between miR-195 and the 3’UTR of *CHEK1* through a luciferase reporter assay (Supplementary Figure 5F,G). Additionally, we generated *CHEK1*-over-expressing H1299 cells and found that over-expression of *CHEK1* confers resistance to paclitaxel and eribulin (Figure 4C, Supplementary Figure 5H,I). We also used two siRNAs to knock-down *CHEK1*. We found that both *CHEK1* siRNAs almost completely eliminate *CHEK1* protein and synergize with paclitaxel and eribulin to repress the growth of NSCLC cells (Figure 4A, D–O). Intriguingly, we also found that a *CHEK1* inhibitor, PF-477736, synergizes with eribulin, but not paclitaxel, in NSCLC cells (Supplementary Figure 6).

Initially, we posited that inhibition of *CHEK1* synergizes with MTAs because MTAs activate *CHEK1* in NSCLC cells. We did not, however, observe any increase of *CHEK1* protein or phosphorylation of *CHEK1* following treatment with paclitaxel or eribulin, as measured by western blot (Figure 4B). We observed downregulation of *CHEK1* protein or phosphorylation of *CHEK1* protein by *CHEK1* inhibitor PF-477736 (10 μ M) in H1299 and H358 cells. However, similar results were not observed in H1993 cells, due to the relative resistance of H1993 cells to PF-477736, as shown in Supplementary Figure 6C. As previously demonstrated, *CHEK1* inhibitor PF-477736 represses Chk1 protein and phosphorylation of Chk1 S296 but promotes phosphorylation of Chk1 S345 [35]. This indicates that PF-477736 has different effects on different serine residues in Chk1. Consistent with the report, our results (Figure 4B) show that PF-477736 (10 μ M) represses Chk1 protein. Our results also show that PF-477736 slightly represses phosphorylation of Chk1 S317 in H1299 and H358 but not in H1993 cells. We suspect the reason why PF-477736 does not repress Chk1 S317 in H1993 cells is because H1993 cells are relatively resistant to PF-477736 (Supplementary Figure 6C).

3.4. miR-195/*CHEK1* expression is correlated with NSCLC patient survival

Analysis of gene expression in LUAD and LUSC patients from TCGA showed that *CHEK1* is expressed at higher levels in tumor tissues compared to adjacent normal tissues in 100% (57 out of 57) LUAD patients and 98% (50 out of 51) LUSC patients (Supplementary Figure

7A). *CHEK1* expression is negatively correlated with miR-195 expression in both LUAD and LUSC patients (Supplementary Figure 7B), consistent with the finding that miR-195 directly targets CHEK1 in NSCLC. Accordingly, the ratio of miR-195 expression to *CHEK1* expression in tumors is much lower than in adjacent normal tissues (Supplementary Figure 7C). We previously reported that high expression of miR-195 is correlated with better survival in LUAD but not in LUSC patients [18]. Here, we show that high expression of *CHEK1* is associated with worse overall and recurrence-free survival in LUAD patients but not in LUSC patients (Figure 5A,B). Furthermore, LUAD patients with high ratio of miR-195 to *CHEK1* is associated with better overall and recurrence-free survival in LUAD but not LUSC (Figure 5C,D). However, CHEK1 alone might be a better prognostic marker than either miR-195 alone or the ratio of miR-195 to CHEK1 in LUAD (Figure 5).

4. DISCUSSION

Recognizing the important roles of miRNAs in tumorigenesis, the delivery of tumor suppressor miRNAs for cancer treatment – called miRNA replacement therapy – has been proposed as a therapeutic avenue [39]. To identify the miRNAs that regulate both the growth of NSCLC cells and their response to MTAs, we performed a high-throughput screen and identified miR-195 as synergizing with paclitaxel to inhibit the growth of cancer cells in NSCLC. We demonstrated that miR-195 synergizes with both a microtubule stabilizer (paclitaxel) and a microtubule destabilizer (eribulin) in NSCLC cells. Consistently, over-expression of miR-195 sensitizes NSCLC cells to MTAs; while knockout of miR-195 contributes to resistance to MTAs. Importantly, tumors with miR-195 over-expression are more sensitive to eribulin treatment, indicating a potential application of miR-195 as a prognostic indicator of treatment response. In addition, induced expression of miR-195 in tumors not only represses tumor growth but also potentiates the efficacy of eribulin in repressing tumor growth. Given that miR-195 is only moderately induced in these tumors, we expect that specific delivery of high concentrations of miR-195 mimic into tumors could further improve the efficacy of eribulin.

Aberrant activation of *CHEK1* has been shown to promote tumorigenesis and to confer resistance to chemotherapy, therefore making it a potential target for cancer treatment [40, 41]. Here, we confirm previous reports that miR-195 directly targets *CHEK1* in NSCLC [14], and reveal that miR-195 synergizes with MTAs through its regulation of *CHEK1*. Knock-down of CHEK1 by siRNA synergizes with paclitaxel and eribulin, while CHEK1 over-expression triggers resistance to paclitaxel and eribulin in NSCLC cells. Additionally, analysis of expression and survival data from TCGA NSCLC cohorts demonstrate that *CHEK1* expression is increased in tumors compared to adjacent normal tissues and that higher expression of *CHEK1* is correlated with worse recurrence-free and overall survival in lung adenocarcinoma patients. The expression of *CHEK1* is negatively correlated with the expression of miR-195 in both lung adenocarcinoma and squamous cell carcinoma tissues. The ratio of miR-195 to *CHEK1* in tumor tissues is significantly associated with recurrence-free and overall survival of lung adenocarcinoma patients, indicating that the miR-195/*CHEK1* axis can be a prognostic biomarker for lung adenocarcinoma and a potential target for treatment. In summary, our work highlights the importance of the miR-195/*CHEK1* axis and provides a new perspective for developing treatments specific for lung adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1. miR-195 sensitizes non-small cell lung cancer (NSCLC) to microtubule-targeting agents *in vitro* and *in vivo*
2. Repression of miR-195 contributes to resistance of NSCLC to MTAs *in vitro*
3. miR-195 regulate the response of NSCLC to MTAs by targeting checkpoint kinase 1 (CHEK1)

Figure 1. A high-throughput screen identified miR-195 as synergizing with MTAs in NSCLC
(A) Experimental design for the HTS screen. **(B)** 44 miRNAs were identified as regulating both the proliferation of NSCLC cells and their response to paclitaxel. **(C)** Ratio of miRNA level in tumor tissues to adjacent normal tissues in lung adenocarcinoma and squamous cell carcinoma patients. **(D)** Evaluation of the interaction between miR-195 and paclitaxel in H1299 cells. **(E)** Evaluation of the interaction between miR-195 and paclitaxel in H1993 cells. **(F)** Evaluation of the interaction between miR-195 and paclitaxel in H358 cells. **(G)** Evaluation of the interaction between miR-195 and eribulin in H1299 cells. **(H)** Evaluation of the interaction between miR-195 and eribulin in H1993 cells. **(I)** Evaluation of the interaction between miR-195 and eribulin in H358 cells. The figures in **(D–I)** show dose-response curves of miR-195, paclitaxel/eribulin, or their combination in NSCLC cells. Predicted additivity for combining miR-195 with paclitaxel/eribulin was based on the Bliss independence model. The tables in **(D–I)** show the combination index (CI) for miR-195 and paclitaxel/eribulin at various values of Fa (fraction affected, which is also the percentage inhibition of cell proliferation) in NSCLC cells.

Figure 3. miR-195 potentiates the efficacy of eribulin in repressing lung tumor growth
(A) Tumor growth curves of nude mice bearing H1299/Control or H1299/miR-195 xenografts treated with vehicle or eribulin (0.5 mg/kg). **(B)** miR-195 levels in H1299/Control or H1299/miR-195 xenografts treated with vehicle or eribulin. **(C)** Body weight changes of mice bearing H1299/Control or H1299/miR-195 xenografts treated with vehicle or eribulin (0.5 mg/kg). **(D, G)** Growth curves of H1299/pTet-miR-195 #3 and #7 xenografts treated with doxycycline, eribulin, or the two together. **(E, H)** miR-195 levels in H1299/pTet-miR-195 #3 and #7 tumors treated with doxycycline, eribulin, or the two together. **(F, I)** Body weight changes of mice under different treatments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 4. Modulation of CHEK1 expression alters the response of NSCLC to MTAs

(A) Protein level of Chk1 after knockdown or over-expression. EV, H1299 cells transfected with empty vector. CHEK1, CHEK1-overexpressing H1299 cells. (B) Protein levels of Chk1 and phospho-Chk1 after treatment with paclitaxel (5nM), eribulin (5nM) or PF-477736 (10uM). (C) Over-expression of CHEK1 confers resistance to paclitaxel and eribulin. (D) Interaction of paclitaxel with siCHEK1 #1 in H1299 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (E) Interaction of paclitaxel with siCHEK1 #1 in H1993 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (F) Interaction of paclitaxel with siCHEK1 #1 in H358 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (G) Interaction of paclitaxel with siCHEK1 #2 in H1299 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (H) Interaction of paclitaxel with siCHEK1 #2 in H1993 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (I) Interaction of paclitaxel with siCHEK1 #2 in H358 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (J) Interaction of eribulin with siCHEK1 #1 in H1299 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). (K) Interaction of eribulin with siCHEK1 #1 in H1993 cells,

evaluated by Bliss independence (figure) and Loewe additivity (table). **(L)** Interaction of eribulin with siCHEK1 #1 in H358 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). **(M)** Interaction of eribulin with siCHEK1 #2 in H1299 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). **(N)** Interaction of eribulin with siCHEK1 #2 in H1993 cells, evaluated by Bliss independence (figure) and Loewe additivity (table). **(O)** Interaction of eribulin with siCHEK1 #2 in H358 cells, evaluated by Bliss independence (figure) and Loewe additivity (table).

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Figure 5. miR-195/*CHEK1* is correlated with patient survival in lung adenocarcinoma
(A) 5-year recurrence-free and overall survival curves of lung adenocarcinoma (LUAD) patients based on *CHEK1* expression. **(B)** 5-year recurrence-free and overall survival curves of lung squamous cell carcinoma (LUSC) patients based on *CHEK1* expression. **(C)** 5-year recurrence-free and overall survival curves of LUAD patients based on the ratio of miR-195 to *CHEK1* in tumor tissues. **(D)** 5-year recurrence-free and overall survival curves of LUSC patients based on the ratio of miR-195 to *CHEK1* in tumor tissues.