MiR-195 suppresses non-small cell lung cancer by targeting CHEK1

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ABSTRACT

MiR-195 suppresses tumor growth and is associated with better survival outcomes in several malignancies including non-small cell lung cancer (NSCLC). Our previous study showed high miR-195 plasma levels associated with favorable overall survival of non-smoking women with lung adenocarcinoma. To further elucidate role of miR-195 in NSCLC, we conducted in vitro experiment as well as clinical studies in a cohort of 299 NSCLC samples. We demonstrated that miR-195 expression was lower in tumor tissues and was associated with poor survival outcome. Overexpression of miR-195 suppressed tumor cell growth, migration and invasion. We discovered that CHEK1 was a direct target of miR-195, which decreased CHEK1 expression in lung cancer cells. High expression of CHEK1 in lung tumors was associated with poor overall survival. Our results suggest that miR-195 suppresses NSCLC and predicts lung cancer prognosis.

INTRODUCTION

Lung cancer is the most common malignancy and leading cause of cancer death in the world [1]. Non-small cell lung cancer (NSCLC) is a major class of lung cancer in which adenocarcinoma and squamous cell carcinoma account for the majority of tumor histology. Despite extensive research and significant improvement in early detection and treatment options, the disease is still difficult to treat and many patients develop recurrent diseases after surgery. Less than 15% of the patients with advanced disease can survive 5 years after diagnosis [2, 3]. For these reasons, many recent studies focused on finding new prognosis biomarker and pivotal molecular associated with development and metastases of lung cancer [4, 5].

MicroRNAs (miRNAs) are small (18 to 24 nucleotides in length), single-stranded, endogenous non-coding RNAs that regulate gene expression post-transcriptionally. Mammalian miRNAs are generally encoded in the introns of pre-messenger RNAs (pre-mRNAs) or 3’ untranslated regions of messenger RNAs (mRNAs). They suppress gene expression by binding to the complementary regions of mRNAs, which either blocks translation or facilitates mRNA degradation through the RNA-induced silencing complex [6]. Recent reports have summarized that aberrant expression of miRNAs has been associated with carcinogenesis and tumor progression [7, 8].

MiR-195 is a member of the miR-15/16 family, which consists of a group of miRNAs (miR-195, miR-15a, miR-15b, miR-16-1 and miR-16-2) that share a similar seed sequence [9]. The sequence of mature miR-195 is conserved across mammalian species [10]. Previous studies have shown aberrant miR-195 expression in multiple cancer sites, including breast cancer [11], hepatocellular carcinoma [12], colorectal cancer [13, 14], gastric cancer [15] and NSCLC [16]. Like other members of the miR-15/16 family, miR-195 has been reported to have different, sometime conflicting, effects on cell growth and apoptosis in cancer. The role of miR-195 in NSCLC, however, remains unclear. In one of our previous studies, we investigated levels and clinical
implications of several miRNAs in the circulation of non-smoking women with lung adenocarcinoma [17], and found high plasma levels of miR-195 associated with better overall survival. These observations led us to further investigate the role of miR-195 in NSCLC. In this investigation, we first confirmed that miR-195 expression was low in NSCLC compared to adjacent non-tumor tissues and low expression was associated with poor prognosis. We then showed in our in vitro experiments that increasing miR-195 expression in lung cancer cells suppressed cell proliferation, migration and invasion. We also identified a target of miR-195, CHEK1, and demonstrated that miR-195 down-regulated its expression and delayed cell cycle progression in lung cancer cells.

RESULTS

Patient characteristics

Clinical features of NSCLC patients in this study are summarized in Table 1. Patient median follow-up time was 35.2 months (range between 0.6 and 82.8 months), and mean age at diagnosis was 61 years old, ranging from 34 to 83 years. Patient median overall survivals

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number (%)</th>
<th>Death</th>
<th>MST (M)</th>
<th>Log-rank P</th>
<th>HR (95% CI)</th>
<th>HR* (95% CI)</th>
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<tr>
<td>Age at diagnosis (n = 299)</td>
<td></td>
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<tr>
<td>&lt; 60</td>
<td>138 (46.15%)</td>
<td>58</td>
<td>65.47</td>
<td><strong>0.011</strong></td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>≥ 60</td>
<td>161 (53.85%)</td>
<td>86</td>
<td>35.47</td>
<td></td>
<td>1.54 (1.10–2.15)</td>
<td>1.62 (1.11–2.36)</td>
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<tr>
<td>Gender (n = 299)</td>
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<tr>
<td>Male</td>
<td>178 (59.53%)</td>
<td>93</td>
<td>52.17</td>
<td>0.164</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>Female</td>
<td>121 (40.47%)</td>
<td>51</td>
<td>52.70</td>
<td>0.78 (0.56–1.11)</td>
<td>0.89 (0.53–1.50)</td>
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<td>Smoking history (n = 299)</td>
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<tr>
<td>No</td>
<td>119 (39.80%)</td>
<td>52</td>
<td>52.70</td>
<td>0.270</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>Yes</td>
<td>180 (60.20%)</td>
<td>92</td>
<td>52.17</td>
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<td>1.21 (0.86–1.71)</td>
<td>0.85 (0.48–1.49)</td>
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<td>Histology subtype (n = 299)</td>
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<tr>
<td>SCCa</td>
<td>142 (47.49%)</td>
<td>74</td>
<td>53.50</td>
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<td>1.00</td>
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<tr>
<td>ADCb</td>
<td>121 (40.47%)</td>
<td>48</td>
<td>72.00</td>
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<td>0.68 (0.47–0.97)</td>
<td>0.52 (0.33–0.83)</td>
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<td>Othersc</td>
<td>36 (12.04%)</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<td>TNM stage (n = 298)d</td>
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<td>I + II</td>
<td>188 (63.09%)</td>
<td>68</td>
<td>44.70</td>
<td><strong>&lt; 0.001</strong></td>
<td>1.00</td>
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<td>III + IV</td>
<td>110 (36.91%)</td>
<td>75</td>
<td>24.47</td>
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<td>2.58 (1.85–3.60)</td>
<td>2.74 (1.89–3.97)</td>
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<td>Low expression</td>
<td>39 (45.88%)</td>
<td>26</td>
<td>16.26</td>
<td><strong>0.025</strong></td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>High expression</td>
<td>46 (54.12%)</td>
<td>23</td>
<td>52.70</td>
<td></td>
<td>0.53 (0.30–0.93)</td>
<td>0.44 (0.24–0.81)</td>
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<td>CHEK1 (n = 276)</td>
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<td>Low expression</td>
<td>155 (56.16%)</td>
<td>70/85</td>
<td>65.46</td>
<td><strong>0.037</strong></td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>High expression</td>
<td>121 (43.84%)</td>
<td>63/58</td>
<td>35.86</td>
<td></td>
<td>1.44 (1.02–2.02)</td>
<td>1.42 (1.00–2.02)</td>
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</table>

aSCC: squamous cell carcinoma.
bADC: adenocarcinoma.
cNo statistics are computed because of no death among these cases.
dNumbers do not equal to the total number due to missing data.
eMST (M): medium survival time (months).
fAdjusted for gender, age at diagnosis, smoking history, family history, histological type, and TNM stage.
were significantly different by age at diagnosis (shorter in older patients), histological type (shorter in squamous cell carcinoma) and disease stage (shorter in advanced stage) (Table 1). Of these patients, tumor expression of miR-195 and CHEK1 were analyzed in 85 and 276, respectively.

**MiR-195 expression in NSCLC**

In analysis of 48 paired tumor and adjacent non-tumor tissue samples, we found miR-195 expression was significantly higher in tumor tissues than in adjacent non-tumor tissues (p < 0.0001; Figure 1A). Survival analysis showed that miR-195 expression in 85 tumor samples was significantly associated with the overall survival of NSCLC patients. Patients with high miR-195 expression had better overall survival compared to those with low expression (p = 0.025; Figure 1B), and the hazard ratio (HR) was 0.53 (p = 0.025), a nearly 50% reduction in risk for death. This association remained significant after adjusting for TNM stage, histology, smoking history, and family history of cancer (HR, 0.44; 95%CI, 0.24–0.81) (Table 1).

**Effects of miR-195 expression on cell proliferation**

After transfecting lung cancer cells (A549, H1299 and H1975) with miR-195 mimic or scrambled miRNA control (miR-NC), we quantified cell numbers with the MTT assay. The results showed that compared to the miRNA control, the number of viable cells was clearly reduced overtime in all 3 cell lines transfected with miR-195 mimic (p < 0.05) (Figure 2A, 2B and 2C), suggesting that cell proliferation was significantly suppressed by miR-195. Furthermore, using flow cytometry to assess cell-cycle status, we found that cells transfected with miR-195 had increased cell numbers in the G1 and G2 phases, but reduced numbers in the S phase (Figure 2D).

**Effects of miR-195 expression on cell migration and invasion**

To assess the effect of miR-195 on cell migration and invasion, we performed the wound healing and transwell assays on cancer cells transfected with miR-195 mimic or with miR-NC. The wound healing assay showed that miR-195 expression appeared to inhibit cell migration in H1975, A549 and H1299 cells (Figure 3A). In the transwell assay, increased miR-195 expression could reduce cell invasion in all three cancer cell lines (Figure 3B and 3C).

**CHEK1, a miR-195 target in NSCLC cells**

Using three miRNA databases, we identified a putative miR-195-binding site located in the 3′-UTR of CHEK1 mRNA (Figure 4A). To further validate the association between miR-195 and CHEK1, we analyzed the TCGA dataset and the result showed that the inverse correlation between miR-195 and CHEK1 was significant (r = –0.46, p < 0.0001) in NSCLC samples (Figure 4B). To confirm if miR-195 directly binds to this location in CHEK1, we cloned a full-length CHEK1 3′-UTR and inserted it into a luciferase reporter vector, downstream from the firefly luciferase gene. As for control, we made a mutant CHEK1 3′-UTR clone which had a 7-nucleotide deletion in the miR-195 binding site (Figure 4C), and the mutant clone was inserted into the same vector. Both vectors were transfected into lung cancer cells A549 and H1299 together with miR-195 or miR-NC. Our experiments showed that miR-195 significantly suppressed the luciferase activity

![Figure 1: MiR-195 expression in NSCLC and association with survival.](image-url)
in the CHEK1 wild type clone compared to miR-NC, but not in the mutant one (Figure 4D), suggesting that miR-195 directly binds to the 3′-UTR of CHEK1 mRNA. The transfection efficiency was examined with RT-qPCR, and our evaluation confirmed that miR-195 expression was increased substantially in the cell lines transfected with miR-195 mimic, but not in those with miR-NC (Figure 4E).

To further confirm the effect of miR-195 on CHEK1, we analyzed protein levels of CHEK1 by western blot in A549, H1299 and H1975 cells transfected with miR-195 or miR-NC. As known targets of miR-195, levels of cyclin D1 and cyclin E proteins were also measured in the cell lines using the western blot. Our protein analyses showed that all these proteins, CHEK1, cyclin D1 and cyclin E, were declined in the miR-195 transfected cell lines compared to the negative controls (Figure 4F), suggesting that miR-195 not only directly bind to CHEK1, lowering mRNA expression, but also down-regulate its protein level along with other molecules involved in cell cycle regulation.

CHECK1 expression and NSCLC survival

To assess if CHECK1 expression in NSCLC was associated with patient survival, we measured CHECK1 expression in 276 tumor samples with immunohistochemical staining (IHC). Results of representative tumor samples stained with high and low CHECK1 expression are shown in Figure 5A and 5B. CHECK1 expression was slightly higher in tumor than in adjacent non-tumor tissues, but the difference was not statistically significant (p = 0.832) (Data not shown). Compared to low expression, however, high CHECK1 expression was significantly associated with poor overall survival (p = 0.037) (Figure 5C). Cox regression analysis confirmed that CHECK1 was associated with survival after adjusting for confounding variables (HR, 1.42; 95% CI, 1.00–2.02) (Table 1). No statistically significant associations were found between CHECK1 expression and clinicopathological features of NSCLC (Supplementary Table 1).
DISCUSSION

Our tissue analysis showed that miR-195 expression was lower in tumor than in adjacent non-tumor tissues and low expression in tumor tissues was associated with unfavorable overall survival of NSCLC patients. These observations were in agreement with our previous finding of high circulating miR-195 being associated with favorable prognosis of non-smoking women with NSCLC [17]. Our in vitro experiments suggest that miR-195 plays an obviously suppressive role in tumor cell proliferation, migration and invasion. Our bioinformatic analysis and cell culture experiments further indicate that the tumor suppressing effects of miR-195 were mediated in part through its down-regulation of CHEK1 mRNA, a cell cycle modulator. We also found that high levels of CHEK1 protein in tumor tissues were associated with poor NSCLC survival. To our knowledge, this is the first study to demonstrate the post-transcriptional regulation of CHEK1 by miR-195 in NSCLC, and their associations with patient survival.

Although two studies reported that miR-195 expression was increased in chronic lymphocytic leukemia and breast cancer [18, 19], most previous studies showed decreased miR-195 expression in several types of cancer, including gastric cancer, breast cancer, colon cancer,
hepatocellular carcinoma, adrenocortical carcinoma and squamous cell carcinoma of the tongue [12, 20–24]. More recently, Luo et al [11] reported low expression of miR-195 in breast cancer specimens compared to adjacent non-tumor tissues, suggesting that the miRNA functions as a tumor suppressor. Another recent study examined miRNA signature in bladder cancer (BC) using deep sequencing. The study demonstrated that miR-195 was down-regulated in BC and could inhibit BC cell proliferation, migration and invasion [25]. These findings were consistent with the observations we made in our clinical studies and cell culture experiments.

Our in vitro experiments showed that increased miR-195 expression could inhibit cell proliferation, suppress cell migration and invasion, and possibly induce cell cycle arrest in the G1 or G2 phases. Similar observations have been reported in a number of cancer cells including NSCLC [16, 26, 27]. The expression of CHEK1 can be regulated by miR-195 in cardiomyocytes and human epidermoid carcinoma cell line reported by

Figure 4: MiR-195 expression and effects on CHEK1 as a target of miR-195. MiR-195 bound directly to the CHEK1 3′-UTRs and down-regulated its expression along with other proteins. (A) A Venn diagram shows 3 software which predict miRNA targets and identified 84 candidate genes which may interact with miR-195. (B) A significant inverse correlation was found in NSCLC between miR-195 and CHEK1 expression in TCGA. (C) A putative miR-195-binding site exists in the 3′-UTR of the CHEK1 mRNA, and 7-nucleotide deletion were generated in the binding site. (D) Transfection of miR-195 inhibited the firefly luciferase activity of the pMIR-REPORT-3′-UTR-CHEK1 (wt), but such inhibition was absent for the reporter which had deletion in the miR-195-binding site (del). MiR-NC was used as a negative control in all the experiments. The impact of miR-195 on CHEK1 expression was normalized and compared to those of negative miRNA (n = 3, p < 0.001). (E) The expression of miR-195 determined by RT-qPCR in three NSCLC cell lines was significantly increased following miR-195 transfection. (F) The protein level of CHEK1 was decreased in three NSCLC cell lines when transfected with miR-195 with beta-actin as a loading control. Two positive control Cyclin D1 and Cyclin E were detected as known targets of miR-195.
two groups respectively [28, 29]. However, no studies has yet examined that CHEK1 is a potential target of miR-195 in NSCLC. Our investigation not only demonstrated that miR-195 interacted with CHEK1 mRNA and suppressed its protein expression in cancer cells, but also showed that CHEK1 expression was associated with patient survival and the direction of the association was consistent with the action of miR-195. CHEK1 encodes a serine/threonine kinase (also known as Chk1) which is a central component of the DNA damage response. CHEK1 regulates cell cycle checkpoints, and coordinates cellular activities involving DNA repair and cell cycle arrest [30]. In our study, we found that high expression of CHEK1 was associated with poor prognosis of NSCLC; similar associations were observed in ovarian cancer [31, 32].

CHEK1 has been considered a potential target for cancer therapy. CHEK1 inhibitors have been tested as therapeutic agents for several types of cancer including lung cancer, and the test results show that the inhibitors may affect the sensitivity of radiotherapy and

Figure 5: CHEK1 protein expression measured by immunohistochemical staining in tissue microarray and its association with lung cancer survival. (A, B) Examples of NSCLC tissue samples stained with H&E or specific CHEK1 antibody under low (50× ) and high (400× ) power microscope; low CHEK1 expression was shown in panel A and high was in panel B (Scale bars represent 200 μm and 50 μm, respectively). (C) Kaplan–Meier overall survival curves according to low and high CHEK1 protein expression in 276 cases. Green line represents low protein expression, and red line represents high protein expression.
chemotherapy [33–36]. Studies also suggest that miR-195 and CHEK1-related signal pathways have been involved in the sensitivity of chemotherapy to breast cancer [37], laryngeal cancer [38] and colon cancer [13]. So far, CHEK1 inhibitors have shown promise in several preclinical models, and have been tested in a number of ongoing or completed phase I and II clinical trials [39–42]. Taken together, we speculate that reducing CHEK1 activity and increasing miR-195 expression work in concert suppressing tumor growth.

In addition to CHEK1, miR-195 also regulates cell cycle by targeting other messenger RNAs. Recently, miR-195 is reported to suppress cell cycle and tumorigenesis through the control of molecules involved in the G1/S phase transition [43, 44], including cyclin D1, CDK4, CDK6, and E2F3. In our experiments, we also found that cyclin D1 and cyclin E were down-regulated by miR-195, which was consistent with the observations made by other investigators [11, 44–46]. Earlier experiments also suggest that miR-195 has additional targets, such as MYB [16], BCL-2 [47], IKKa and TAB3 [26].

To evaluate the validity of our findings, we downloaded microRNA and mRNA expression data from the Cancer Genome Atlas (TCGA). Our analysis showed down-regulation of miR-195 (Supplementary Figure 1A) and up-regulation of CHEK1 (Supplementary Figure 1B) in tumor samples in comparison to adjacent tissues. Furthermore, high CHEK1 expression was associated with poor survival of NSCLC patients (p = 0.031) (Supplementary Figure 1D). No association, however, was found between miR-195 and survival (p = 0.208) (Supplementary Figure 1C) in TCGA.

In conclusion, we found that NSCLC had lower miR-195 expression in tumor than in adjacent tissues and low expression was associated with poor overall survival. This finding was consistent with our previous observation of miR-195 in plasma. Our cell culture experiments of lung cancer cells showed that increased miR-195 expression could suppress cell proliferation, migration and invasion. We also found that miR-195 was able to bind to CHEK1 mRNA down-regulating its expression. Low CHEK1 protein was associated with favorable survival of patients with NSCLC. Collectively, high miR-195 and low CHEK1 work synergistically suppressing tumor growth and improving the survival outcome of NSCLC patients.

This investigation has led to several novel observations. First, to our best knowledge, this is the first report showing that miR-195 suppressed NSCLC through, at least partially, down-regulating the expression of CHEK1, a newly discovered target in lung cancer. Furthermore, it is novel to know that miR-195 and CHEK1 can be independent prognostic factors in NSCLC. Thus, of particular interest, the miR-195/ CHEK1 axis might represent a new molecular target for NSCLC treatment.

**MATERIALS AND METHODS**

**Patients and tumor samples**

Patients in the study were recruited from the Tianjin Medical University Cancer Hospital (TMUCH) between May, 2006 and July, 2011. During the time, we recruited 299 newly diagnosed patients who had histologically confirmed non-small cell lung cancer (NSCLC). Each patient provided tissue samples for the study, and the specimens were collected during tumor resection. The tissue samples were histologically confirmed to be tumor or non-tumor tissues, and were stored at −80°C until analysis. All patients enrolled in the study were followed from surgery to August 28, 2013 through scheduled office visits and regular telephone contacts. Information on histology, tumor size, disease stage, lymph node involvement, distant metastasis, and treatments was extracted from patient medical records and pathology reports. Demographic features, tobacco use and family history of cancer were collected using a structured questionnaire. The study was approved by the medical ethics committee at TMUCH.

**RNA extraction and analysis**

Total RNA was extracted from the collected fresh-frozen tissue specimens using the standard Trizol method; microRNA expression was measured with the microRNA assay (Applied Biosystems Inc, US). The expression analysis is described briefly as follows. First, 25 ng of total RNA were reverse-transcribed to cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems Inc, US). The reverse transcription (RT) was completed sequentially under the following conditions: incubation at 16°C for 30 minute, 42°C for 30 minute and 85°C for 5 minute. After RT, quantitative polymerase chain reaction (qPCR) was performed in the 7900 HT-Fast real-time PCR system (Applied Biosystems Inc., US) following the protocol of denaturing at 95°C for 10 minute, and 40 cycles of denaturing at 95°C for 15 second and annealing and elongation at 60°C for 1 minute. The qPCR results were analyzed with the SDS Relative Quantification Software version 2.1 (Applied BioSystems Inc., US). Small RNA RNU6 was utilized as an endogenous control to normalize the quantity of cDNA used for analysis of miR-195 expression. All samples were analyzed in triplicate, and the measurement was repeated if the coefficient of variation was greater than 5%. The expression level of miR-195 was calculated based on the formula 2−ΔΔCt, where ΔCt = Ct (miR-195)−Ct (RNU6).

**Cell culture and transfection**

Three lung cancer cell lines, A549, H1299 and H1975, were selected for our cell culture experiments. These cell lines were maintained in RPMI-1640 (GIBCO, US), supplemented with 10% (V/V) fetal bovine serum.
(FBS) (GIBCO, US), and the culture media were changed every 3 days. For in vitro experiments, cells were seeded at $3 \times 10^5$ per well in the 6-well plates, $5 \times 10^5$ per well in the 24-well plates, and $2 \times 10^5$ per well in the 96-well plates, and allowed to attach for at least 24 hours. To assess the effects of miR-195 on cell activities, 50 nM miR-195 mimic or scrambled miRNA control (miR-NC) (Shanghai GenePharma, China) were transfected to the cells using the Lipofectamine RNAiMAX (Invitrogen, US) according to the manufacturer’s instruction. At 6 hours post-transfection, culture media were replaced with those containing 10% FBS. CHEK1 3′-UTR were cloned into the pMIR-REPORT Luciferase plasmids (Promega, US) which were transfected into the lung cancer cells using the DharmaFECT Transfection Reagent (Thermo, US).

Luciferase reporter assays

A549 and H1299 cells maintained in the 24-well plates were transfected with 0.4 μg of the pMIR-REPORT Luciferase plasmids and miRNA using the Lipofectamine 2000 (Invitrogen, US). The relative luminescences were measured 24 hours post-transfection using the Dual Luciferase Reporter Assay Kit (Promega, US).

MTT assay

Cell proliferation was measured by the MTT assay (Nanjing KeyGEN Biotech, China). After being seeded in the 96-well plates for 24 hours, cells were transfected with miR-195 mimics or miRNA controls. At 24, 48, 72 and 96 hours of post-transfection, cells were gently washed with PBS, and 20 μl MTT (5 mg/ml) were added in the cell culture. After 4 hours of incubation, the media were discarded, and 150 μl DMSO was added in each well to dissolve the precipitates. The absorbance of the resulting solution was measured at 590 nm wavelength with a microplate reader (Bioteck, US). Each experimental condition was carried out in 6 replicates, and repeated 3 times.

Wound healing assay

Cell migration was measured using the wound healing assay in the 6-well plates. A fine line was scraped with a 10 μl tip in each well after cultured cells became fully confluent. After scratch, cells were continuously cultured in the media with 3% FBS for 72 hours. Microscopic pictures of the cultures were taken at 0, 24, 48 and 72 hours. The experiments were performed 3 times independently.

Trans-well assay

To analyze cell migration, cultured cells were re-seeded at a concentration of $5 \times 10^4$ onto the upper chamber of a 8 mm pore size insert (BD, US). The inserts were placed in the wells of a 24-well plate, and the cells were cultured in serum free medium. For invasion analysis, $1 \times 10^5$ cells were added onto the upper chamber of matrigel-coated (BD, US) 8 mm pore size inserts placed in the wells of a 24-well plate, and the cells were cultured in the media containing 10% FBS. After 24 hours of incubation, cells migrated through the membrane or invaded through the matrigel into the lower chamber of the insert were fixed with 75% ethanol for 30 min and stained with crystal violet. After taking photograph from five random visual fields, cells were counted in each photo. These experiments were repeated 3 times.

Cell-cycle analysis

Cultured cells were seeded onto the 6-well plates at a density of $1 \times 10^6$ cells per well and incubated overnight. After 48 hours of transfection, cells were collected and washed with PBS. Cell pellets were fixed in 70% ethanol overnight at −20°C. The fixed cells were washed in PBS and resuspended in the Cell Cycle Reagent (Millipore, US) at $5 \times 10^5$ cells/ml. The cells were incubated in the dark for 15 minutes at room temperature. The cell solutions were analyzed by a flow cytometer, guava easyCyte™ (Millipore, US), to determine cell populations at different cell cycle phases. The DNA contents of the stained cells were analyzed using the Modfit LT software (Verity Software House, US).

Tissue microarray and immunohistochemical staining

CHEK1 protein expression in tumor samples was measured with immunohistochemical staining. Tissue microarrays (TMA) were constructed from the archived formalin-fixed paraffin-embedded tissue blocks using the “TMA Builder” (Beecher Instruments, USA). A total of 15 slides were developed which contained both tumor and adjacent non-tumor tissues from 276 patients with NSCLC. Each tissue sample contributed at least two 0.6 mm cores, and each core represented either tumor or adjacent non-tumor tissue, determined by the study pathologists who examined the corresponding sections stained with hematoxylin & eosin. TMA slides were first stained with a CHEK1 antibody. This primary antibody was then interacted by a secondary antibody conjugated with horseradish peroxidase from the EnVision Detection Systems (DAKO, Denmark). After formation of the antigen-antibody-antibody complex, a substrate of the peroxidase, diaminobenzidine, was added as chromogen. The tissue staining was done according to the manufacturer’s instructions. All the TMA slides were counterstained with hematoxylin. TMA staining results were evaluated independently by two pathologists, and the samples with different results were re-evaluated until a consensus was reached. The staining conditions for TMA were optimized when both positive and negative cells were present in the same tissue sample. Signals were considered...
positive when the reaction products were localized in the expected cellular component. The tissue staining results were scored based on signal distribution (distribution score) and intensity (intensity score). The distribution score includes 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%), which indicates the percentage of positive cells in all the tumor cells present in a sample. The signal intensity consists of 0 (no signal), 1 (weak), 2 (moderate), or 3 (strong). The final staining score was the product of distribution and intensity scores.

**Western blot analysis**

Western blot was performed for detection of specific proteins in our cell culture experiments. The protocol started with uploading 30 μg proteins from the whole cell lysate in each sample onto a 10% PAGE gel. After electrophoresis and gel transferring, the membrane was blocked with 5% non-fat milk in 1× Tris-buffered saline (pH 7.4) containing 0.05% Tween-20, and then probed with primary antibodies at concentrations of 1:1000 for β-actin (Santa Cruz Biotechnology, US), 1:2000 for CHEK1 and Cyclin E and 1:10000 Cyclin D1 (both from Epitomics, US). Secondary antibodies were added at concentrations of 1:10,000 to 1:20,000. The detected proteins were visualized using the Visualizer Western Blot Detection Kit (Millipore, US).

**TCGA data analysis**

To evaluate the validity of our findings, we downloaded microRNA and mRNA expression data and their corresponding clinical information from the Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/tcga/) in August, 2014. The microRNA expression data generated from the Illumina Genome Analyzer and HiSeq 2000 contained 956 NSCLC tumors and 91 normal lung tissue samples. The mRNA expression data generated from the Illumina HiSeq 2000 involved 977 NSCLC tumors and 108 normal lung tissue samples. The expression data were processed with quantile normalization using the preprocessCore in the R/Bioc conductor package [48]. Samples and corresponding clinical data were linked by tumor barcodes. Survival analyses were performed on NSCLC tumor samples that had follow-up information using the SAS software. Pairwise comparisons were evaluated with t-test.

**Statistical analysis**

The study results were analyzed using the statistical software SAS (version 9.1.3, SAS) and SPSS (version 16.0, SPSS Inc.). RNA expression was calculated as expression index (EI) based on the formula \( \frac{2^{-\Delta \Delta Ct}}{2} \). The EI was analyzed as a continuous variable. Independent two-sample Student’s t-test was used to compare the differences in miR-195 expression by clinicopathological features of NSCLC. Kaplan–Meier survival curves were compared between patients with high and low expression of biomarkers using the log-rank test. Associations between molecular markers and NSCLC survival were also examined with the Cox proportional hazards regression model at both univariate and multivariate levels. In the Cox regression analysis, miR-195 and CHEK1 were categorized into high and low groups using their medians as cutoff. Differences were considered statistically significant when a p value was less than 0.05. All p values reported were two-side.

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**CONFLICTS OF INTEREST**

The authors indicate no potential conflict of interest.

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