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ORIGINAL ARTICLE

Suppressed miR-424 expression via upregulation of target gene *Chk1* contributes to the progression of cervical cancer

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MicroRNAs (miRNAs) act as important gene regulators in human genomes and their aberrant expression links to many malignancies. We previously identified a different characteristic miRNA expression profile in cervical cancer from that in cervical normal tissues, including the downregulated miR-424. However, the role and mechanism of miR-424 in cervical cancer still remain unknown. Here, we focused on identifying the tumor-suppressive function and clinical significance of miR-424 and exploring the mechanistic relevance by characterizing its target. We showed a significantly decreased expression of miR-424 in 147 cervical cancer tissues versus 74 cervical normal tissues by performing quantitative RT – PCR. In 147 cervical cancer tissue samples, low-level expression of miR-424 was positively correlated with poor tumor differentiation, advanced clinical stage, lymph node metastasis and other poor prognostic clinicopathological parameters. Further *in vitro* observations showed that enforced expression of miR-424 inhibited cell growth by both enhancing apoptosis and blocking G1/S transition, and suppressed cell migration and invasion in two human cervical cancer cell lines, SiHa and CaSki, implying that miR-424 functions as a tumor suppressor in the progression of cervical cancer. Interestingly, overexpression of miR-424 inhibited the expression of protein checkpoint kinase 1 (Chk1) and phosphorylated Chk1 (p-Chk1) at residues Ser345 and decreased the activity of luciferase-reporter containing the 3′-untranslated region (UTR) of Chk1 with predicted miR-424-binding site. Moreover, miR-424 expression levels were inversely correlated with Chk1 and p-Chk1 protein levels in both cervical cancer and normal tissues. Furthermore, RNAi-mediated knockdown of Chk1 decreased matrix metalloproteinase 9 expression and phenocopied the tumor suppressive effects of miR-424 in cell models. Taken together, our results identify a crucial tumor suppressive role of miR-424 in the progression of cervical cancer at least partly via upreglating the expression of Chk1 and p-Chk1, and suggest that miR-424 might be a candidate of prognostic predictor or an anticancer therapeutic target for cervical cancer patients.

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INTRODUCTION

Cervical cancer ranks as the third most common malignancy in women worldwide, with an estimated incidence of 529 000 new cases per year. More than 85% of the global burden occurs in the developing world, where, in most countries, cervical cancer is the most prevalent cause of cancer-related deaths in women.[1] Despite well organized screening and early treatment programmes have been effective in preventing cervical cancer, they are difficult to be implemented in lower socioeconomic settings and occurrence of invasive cervical cancer is still common. Furthermore, given the limited degree of success with conventional therapies including surgery, radiation and chemotherapy in various settings in an effort to improve disease control and survival in cervical cancer patients, invasive cervical cancer is almost always fatal and the forces driving cervical cancer aggressiveness are still poorly understood. Therefore, identification of molecular aberrations that might be helpful to create novel diagnostic and therapeutic strategies remains an important facet in the current management of this malignancy.

In the past decade, an explosion of research has been done on microRNAs (miRNAs) since their discovery in 1993,[2] which makes the miRNA world expand at vertiginous speed. It is now well known that miRNAs are key regulators of gene expression and modulate up to one-third of all genes though they constitute only about 1% of human genomes.[3–5] In parallel, much hard genetic evidence has showed that many miRNAs are strongly implicated in cancers, either as tumor suppressors or oncogenes,[6] and altered expression of miRNAs could be used as robust and important biomarkers for cancer risk, diagnosis and prognostic prediction, even as miRNA-based therapeutic targets with a great interest. For instance, miR-21 overexpression occurs frequently in most cancers and is linked to increased tumor cell growth.[7,8] In addition, conditional expression of miR-21 *in vivo* gave rise to various clinical signs of hematological malignancies. Meanwhile, the tumors showed rapid regression with knockdown of miR-21.[9] Furthermore, it was observed that overexpression of miR-21 in primary breast cancer tissues was correlated with advanced clinical stage, lymph node metastasis and poor survival of the patients.[10]

Given the importance of miRNAs in cancers, our previous study identified a characteristic miRNA expression profile in cervical carcinoma, in which miR-424 was one of the most obviously

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downregulated miRNAs in the cervical cancer compared with normal cervical tissues.[11] It has been shown that miR-424 controls many crucial biological activities, including cellular differentiation and proliferation, cell-cycle progression and angiogenesis, all of which are often perturbed in malignancies.[12–14] Indeed, aberrant miR-424 expression has been observed in some other cancer types.[15–17] These data highlight the potential pivotal roles of miR-424 both in the development and progression of malignancy. However, the functional characteristics of miR-424 remain largely unknown. Relatively few miR-424-target interactions are experimentally identified and convincing data in sufficient series of well-defined clinical specimens are still lacking.

In the present study, we explored the tumor-suppressive significance of miR-424 both in cervical cancer tissues and cells, and further identified for the first time that cell-cycle checkpoint kinase 1 (Chk1) as a direct functional target mediates multiple suppressive actions of miR-424 on the progression of cervical cancer.

RESULTS

Expression of miR-424 is frequently downregulated in cervical cancer tissues and associated with clinicopathological characters

In our previous miRNA microarray, miR-424 was downregulated to 23.2-fold in cervical cancer tissues compared with normal tissues.[11] Here, we examined miR-424 levels in 147 cervical cancer and 74 cervical normal tissues using quantitative real-time RT–PCR (qRT–PCR). Consistent with the microarray data, expression of miR-424 was also significantly reduced by 4.10-fold ($P = 2.85E\text{-}22$) in cervical cancer tissues compared with normal tissues (Figure 1a).

The associations of miR-424 expression with clinicopathological parameters, including age, clinical stage, pathological grade, SCC-Ag, tumor size, lymph node metastasis, vascular involvement and stromal invasion, were further analyzed in 147 cervical cancer patients. Patients were divided into two groups by the 75th percentiles of $2^{-\Delta\Delta Ct}$ miR-424 low expressers ($n = 110$) and miR-424 high expressers ($n = 37$).[18,19] Correlation between miR-424 expression level and clinicopathological characters of cervical cancer is summarized in Table 1. Remarkably, low miR-424 level was significantly associated with advanced FIGO stage, poor tumor differentiation, nodal metastasis, vascular involvement and deep stromal invasion (all $P < 0.05$), suggesting that downregulated miR-424 expression may be involved in the progression and metastasis of cervical cancer.

Enforced expression of miR-424 functionally suppresses proliferation, induces apoptosis and blocks G1/S transition in cervical cells

Given that miR-424 was underexpressed in primary cancer tissues as well as in cervical cancer cell lines according to other's previous observations,[20,21] we hypothesized that miR-424 might act as a tumor suppressor and restoration of miR-424 expression could affect cell proliferation in cervical cancer cells. Therefore, miRNA mimic or negative control was transfected into two human cervical carcinoma cell lines, Siha and CaSki cells, and then the expression of miR-424 was detected in both kinds of cells at 0, 24, 48, 72 and 96 h posttransfection. SiHa is a local tumor-derived cell line, containing a single HPV-16 viral genome, whereas CaSki is a more aggressive and metastatic cervical cancer cell line, containing about 500 copies of the HPV 16 viral genomes in each cell. The results showed a significant upregulation of miR-424 expression in Siha and CaSki cells transfected with miR-424 mimic compared with the negative control, and the high miR-424 expression level continued for at least 4 days (Supplementary Figure S1). Further, cell proliferation experiments were carried out in both kinds of cells that were transfected with miR-424 mimic or negative control. The growth curve showed a great decrease of cell viability, measured by the MTT assay, in both cell lines transfected with miR-424 mimic compared with negative controls (Figure 1b). Furthermore, miR-424 transfection provoked a robust apoptotic response in SiHa and CaSki cells assessed by an AnnexinV-PI apoptosis detection kit (Biouniquer, San Diego, CA, USA) (Figure 1c). In consistent with apoptosis result, we simultaneously performed the cell-cycle patterns by using BrdU flow kits in SiHa and CaSki cells. Notably, overexpression of miR-424 triggered an accumulation of cells at G1 phase, and decreased the number of cells at S phase and G2/M phase in both SiHa and Caski cells when pulsed with BrdU for 1 h (Figure 1d). Cumulative labeling with BrdU for 12 h, overexpression of miR-424 showed a more significant blocking of cells at G1 phase while G1 phase cells moved favorably through S phase into G2/M phase in the negative control cells (Figure 1e). These results verified that overexpression of miR-424 blocked the G1/S transition. Thus, our findings suggest that miR-424 effectively retards growth by inducing apoptosis and blocking G1/S transition in cervical cancer cells *in vitro*.

Forced miR-424 expression reduces migration and invasion in cervical cancer cells

To verify further miR-424 function on the progression and metastasis of cervical cancer, we determined the effect of miR-424 overexpression on migratory and invasive capacity in cultured cervical cancer cells. An *in vitro* wound healing assay showed that both miR-424 overexpressing SiHa and CaSki cells were less proficient than equivalent miR-NC transduced cells at closing an artificial wound created over the confluent monolayer (24 h, SiHa $P = 7.6E\text{-}07$, CaSki $P = 4.3E\text{-}06$; Figure 2a). Moreover, miR-424 upregulation dramatically impaired the invasive capacity of two cancer cell lines by matrigel chamber assays ($P = 1E\text{-}06$, 6E-08, respectively; Figure 2b). These observations further supported the role of miR-424 expression in antagonizing invasion and metastasis. Taken together, our results first demonstrated that miR-424 acts as an inhibitor on malignant growth, invasion and metastasis in cervical cancer cells, consequently contributing to the progression of cervical cancer.

Chk1 is a direct target of miR-424 in cervical cancer cells

To identify the specific gene target of miR-424 through which miR-424 suppresses biological behavior of cervical cancer cells, we searched public algorithms (TargetScan, PicTar and miRecords) for theoretical target genes whose upregulation could mediate the observed effects of miR-424. Because a recent study suggested that inhibition of Chk1 was cytotoxic, causing a significant inhibition of cell growth and inducing apoptosis, in neuroblastoma,[22] we determined to observe whether Chk1 served as the right target of miR-424 among all the potential candidates, though the biological function of Chk1 remained largely elucidated in cervical cancer. Western blot analysis showed that transfection with miR-424 mimic significantly decreased the levels of Chk1 protein in both SiHa and CaSki cells (Figures 3a and b), but Chk1 mRNA level was not changed (data not shown). Meanwhile, we also detected the levels of phosphorylated Chk1 (p-Chk1) at residues Ser345, representing activated Chk1, and found that p-Chk1 was also consistently reduced in response to the restored expression of miR-424 in two cancer cell lines (Figures 3a and b).

Furthermore, fragments of the Chk1 3′-untranslated region (3′-UTR) containing the miR-424-binding sites were subcloned into the pmiRGLO dual-luciferase reporter vector (Figure 3c), and the reporter vector was then cotransfected with miR-424 mimic or miRNA-negative control. As illustrated in Figure 3d, a significant reduction of luciferase activity was observed in SiHa cells with miR-424 transfection, compared with the negative control.

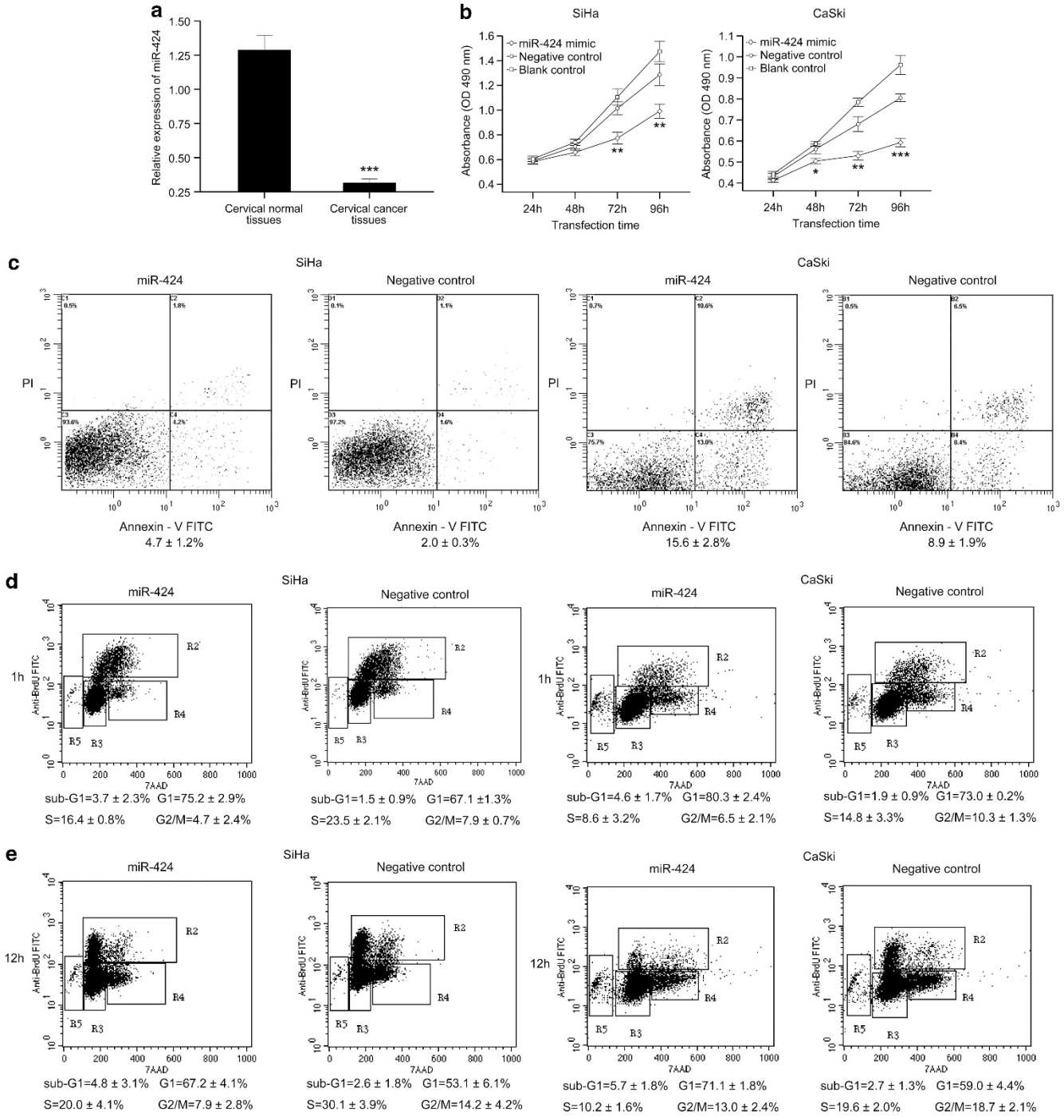

Figure 1. miR-424 expression was underexpressed in cervical cancer tissues compared with cervical normal tissues by real-time PCR analysis and enforced miR-424 expression inhibited cell viability by inducing cell apoptosis and cell-cycle arrest in G1 phase in both SiHa and CaSki cells. Cells were transiently transfected with 50 nM miR-424 mimic or mimic-negative control, respectively. (**a**) miR-424 level was shown as $2^{-\Delta\Delta Ct}$ of cancer versus normal tissues. U6 snRNA was used as internal control and for normalization of the data. Data were presented as mean ± s.e., $^{***}P<0.001$ vs control by nonparametric Mann–Whitney *U*-test. (**b**) At 24, 48, 72 and 96 h after transfection, cell proliferation was determined by the MTT assay. Data were presented as mean ± s.e. from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. (**c**) 72 h after transfection, apoptosis assay was performed to determine the early apoptotic rate of the two cancer cells. Data were the averages of three independent runs with duplicated samples. Mean ± s.d. (**d**, **e**) 48 h after transfection, BrdU was added to the culture media and incubated for 1 and 12 h, respectively, cells were then collected, stained with FITC anti-BrdU and 7-AAD, a DNA stain, and cell cycle was analyzed with FACS. Results were average from three separate experiments. Data were presented as mean ± s.d.

In addition, mutation of the predicted-binding site of miR-424 on the Chk1 3′-UTR rescued the luciferase activity (Figure 3d). Thereby we confirmed the direct interaction of miR-424 with the 3′-UTR of Chk1 mRNA.

Chk1 mediates multiple biological actions of miR-424 in cervical cancer cells

To recognize whether Chk1 serves as a critical mediator of miR-424's role in cervical cancer cells, we knocked down Chk1

Table 1. Association between miR-424 expression and clinicopathological parameters of cervical cancer patients

Characteristic	*Total (n = 147)*	*miR-424 expression*		P
		Low	*High*	
Age, years				0.306
⩽35	17	11	6	
>35	130	99	31	
FIGO stage				**0.038**
IB	108	76	32	
IIA	39	34	5	
Pathological grade				**0.016**
Grade 1	3	1	2	
Grade 2	131	96	35	
Grade 3	13	13	0	
SCC-Ag, ng/ml				0.115
<4	128	93	35	
⩾4	19	17	2	
Tumor size, cm				0.322
<4	119	87	32	
⩾4	28	23	5	
Lymph nodes metastasis				**0.038**
Negative	123	88	35	
Positive	24	22	2	
Vascular involvement				**0.032**
Negative	98	68	30	
Positive	49	42	7	
Deep stromal invasion				**0.004**
<66%	90	60	30	
⩾66%	57	50	7	

Abbreviations: Ag, antigen; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma. Bold values were presented as $P<0.05$.

using specific RNAi in SiHa and CaSki cells. Western blot analysis showed that Chk1 siRNA reduced Chk1 and p-Chk1 protein levels by ~5-fold and ~3-fold, respectively (Figure 4a). As expected, siRNA against Chk1 produced an anti-proliferative effect compared with non-targeting siRNA control (Figure 4b). Moreover, both SiHa and Caski cells transfected with Chk1 siRNA presented enhanced cell apoptosis (Figure 4c), accompanied by a cell-cycle arrest at the G1/S transition (Figures 4d and e). In addition, abolition of Chk1 expression inhibited the migratory potential of both cell lines to an extent similar to that seen with miR-424 overexpression ($P = 1.0E-04$ and 1.9E-07, respectively) (Figure 5a). Similarly, siRNA specific for Chk1 suppressed invasive behavior of both cell lines ($P = 4.4E-05$ and 3.0E-07, respectively; Figure 5b) to a degree comparable to that caused by miR-424 overexpression. Together with the observed miR-424 inhibitive effect on Chk1, our results suggest that Chk1 as a direct target mediates multiple biological actions of miR-424 in cervical cancer cells.

Chk1 regulates cell metastasis-related protein matrix metalloproteinase 9 (MMP9)

Worth of note, inhibition of Chk1 expression decreased cell migration and invasion in cervical cancer cells. However, the mechanistic link between Chk1 and cell invasion remains elusive. It is well known that proteolytic degradation of basement membrane and extracellular matrix is a crucial event in tumor invasion and metastasis.[23] MMP9 is of particular important because upregulation of MMP9 expression has been reported to contribute to metastasis in cervical cancer.[24,25] Our experiments showed the evidence of the associations of Chk1, p-Chk1 and MMP9 with metastasis in four cervical cancer tissues with lymph node metastasis and eight without by western blot. The expression of three proteins exhibited a significant increase in cervical cancer tissues with lymph node metastasis (Figure 5c), and the expression of Chk1 protein was positively correlated to MMP9 expression with a correlation coefficient of 0.845, and p-Chk1 to MMP9 with a correlation coefficient of 0.842 (Table 2). Furthermore, we assessed the abolition of Chk1 expression on MMP9 expression in SiHa and CaSki cells. The siRNA specific for Chk1 significantly decreased the level of MMP9 protein expression compared with negative controls in both cell lines (Figure 5d). Thus, our results suggest that Chk1 regulates cell invasion and metastasis through upregulating MMP9 in the cervical cancer.

Chk1 and p-Chk1 protein are upregulated in cervical cancer tissues and inversely correlated with miR-424 expression

To verify the inhibition of miR-424 on Chk1 and p-Chk1 expression *in vivo*, we examined the levels of Chk1 and p-Chk1 expression by immunohistochemistry (IHC) staining in 147 cervical cancer and 74 normal tissues that were the same cases for miR-424 detection by qRT–PCR. Concomitantly, expression of Chk1 and p-Chk1 were also examined by western blot in an extended panel of 40 cervical cancer samples and 20 normal tissues. As shown in Figure 6, both IHC staining and western blot showed that Chk1 and p-Chk1 protein levels were visibly elevated in cervical cancer tissues with average value of 5 and 5.6 compared with the normal tissue as 1.0, respectively. The expression of Chk1 or p-Chk1 protein was inversely correlated with miR-424 expression in 221 cervical tissues with a correlation coefficient of −0.74 or −0.754 in normal tissues and −0.514 or −0.487 in cervical tissues, respectively (Table 3). We then analyzed the association of Chk1 and p-Chk1 expression with clinicopathological parameters in cervical cancer patients, and found significantly increased expressions of Chk1 and p-Chk1 in patients with poor prognostic factors, as similar as miR-424 low expression (Table 4). These reciprocal correlations were particularly striking and confirmed from the other side that Chk1 as a direct target participates in the procedure in which miR-424 suppressed tumor progression in the cervical cancer.

DISCUSSION

It has been well known that some miRNAs have key roles in carcinogenesis or development in various cancers. Identification of a specific miRNA contributing to tumor invasion and progression may offer a biomarker for predicting prognosis or a target for therapy of the disease. The alteration of miR-424 expression level in cervical cancer was observed in three independent studies using miRNA array or deep sequencing,[11,20,21] but the role, mechanism and clinical significance of miR-424 downregulation in cervical cancer have not been known. In this study, we explored, for the first time to our knowledge, the potential suppressive roles and the regulatory mechanisms of miR-424 by directly targeting Chk1 in the development and progression of cervical cancer through tissue samples, clinical data and cell models.

Previous studies have shown that miR-424 may act as a potential tumor suppressor miRNA.[15,16,26,27] For instance, a decreased aberrant miR-424 expression is accompanied by a potent suppression of oncogene PLAG1 in chronic lymphocytic leukemia.[16] However, there are also some opposite findings. It was observed that miR-424 expression was higher in Epstein–Barr virus-associated B-cell lymphoma, squamous cell carcinoma of the tongue and colonic cancer.[17,28,29] This discrepancy may be related to different

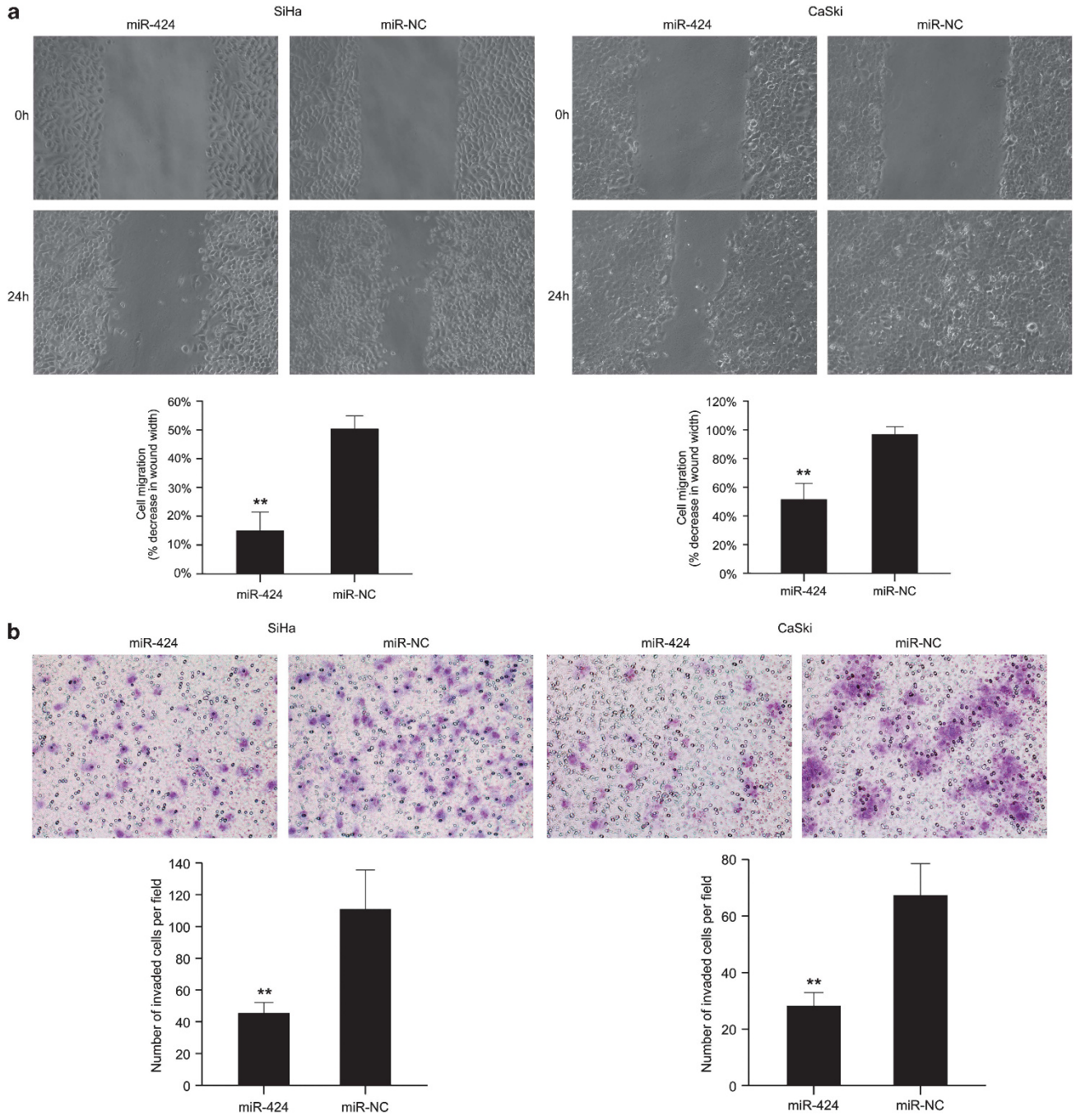

Figure 2. miR-424 inhibited cervical cancer cell migration and invasion. (**a**) Wound healing assay was performed in SiHa and CaSki cells (1×10^6, 6-cm plate) transfected with either miR-424 mimic or negative control (miR-NC; 50 nM), respectively. The wound healing was determined at the time points as indicated. Bars represented the percentage of wound healing. Columns, average of three independent measurements; bars, s.d. (**b**) Invasion activities were measured in SiHa and CaSki cells with transwell chambers, as described in Materials and methods. Photos were representative fields of invasive cells on the membrane. Magnification, $\times$ 200. Bar graphs showed the average number of cells per field on the underside of the membrane $\pm$ s.d. $^{**}P < 0.01$.

actions of the same miRNA on different kinds of cancer. Here, we identified miR-424 to be significantly downregulated by 4.1-fold in cervical cancer tissues compared with cervical normal tissues by large-scale samples. Moreover, we uncovered a parallel association of low miR-424 expression with poor prognostic factors, such as advanced clinical stage, poor tumor differentiation, lymph node metastasis, vascular involvement and deep stromal invasion, suggesting that miR-424 may have a suppressive role on progression of cervical cancer. Consistent with clinical data, restoration of miR-424 expression in cervical cancer cells remarkably affected cell biological behaviors, including suppressed proliferation, enhanced apoptosis, blocked G1/S transition, and inhibited migration and invasion. Thus, our *in vitro* and *in vivo* findings together suggest that miR-424 functions as s tumor suppressor and inhibition of miR-424 expression contributes, by inducing or promoting malignant cellular behaviors, to the progression of cervical cancer, and even may predict a poor prognosis of the patients.

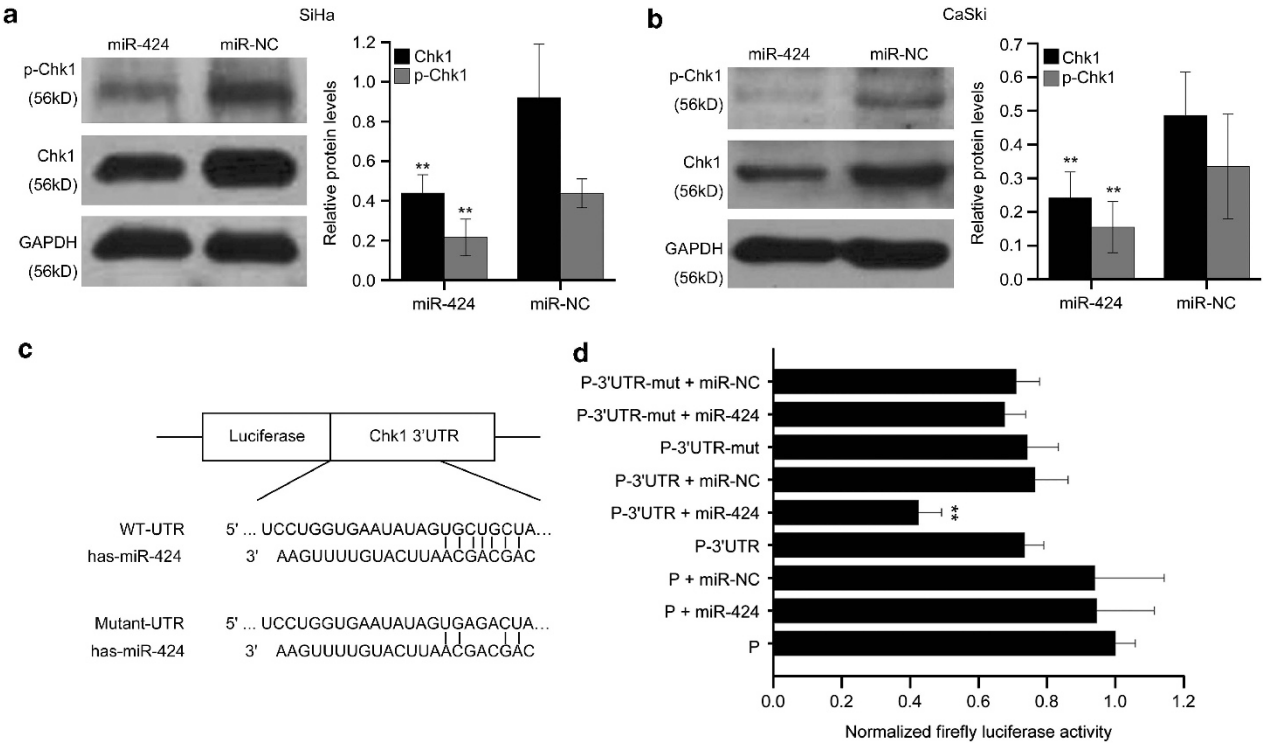

Figure 3. miR-424 suppressed Chk1 and p-Chk1 expression and directly targeted Chk1 3′-UTR. (**a**, **b**) 72 h after transfection with 100 nM of miRNA mimic or mimic control, the endogenous protein levels of Chk1 and p-Chk1 (ser 345) in SiHa and CaSki cells were detected by western blot. Bars indicated the relative protein levels that were normalized to GAPDH. Data were presented as mean ± s.d. ($n = 3$). (**c**) Putative miR-424-binding site in the Chk1 3′-UTR. Mutation was generated in the Chk1 3′-UTR sequence by mutating 3nt that is recognized by miR-424. Either wild-type (WT) or mutant Chk1 3′-UTR was subcloned into the dual-luciferase reporter vector. (**d**) The luciferase reporter vector containing WT Chk1 3′-UTR or mutant Chk1 3′-UTR was cotransfected into SiHa cells with miR-424 mimic or miRNA mimic-negative control. Firefly luciferase activities were determined 48 h posttransfection and normalized to Renilla luciferase. The relative luciferase activity (luminescence) was obtained after normalizing with the control empty PmiRGLO plasmid. Each column represented the mean ± s.d. of three independent experiments. $^{**}P < 0.01$.

Although the suppressive role of miR-424 in the progression of cervical cancer was explored, a lack of knowledge regarding miRNA target would hamper a full understanding of the biological functions of the aberrant miRNA expression. To gain a further insight into the mechanisms involved in the miR-424-induced inhibition on cervical cancer progression, we used the available computative approaches to predict gene targets for miR-424.[30,31] Although multiple cancer-associated genes were predicted by at least two of the software applications, Chk1 was selected as a candidate target of miR-424 for further research because of its promoting function as previous study.[22] We found that the levels of Chk1 and p-Chk1 protein, activated Chk1 with phosphorylated residue Ser345, were inversely correlated with miR-424 levels in cervical cancer and normal tissues. Chk1 and p-Chk1 expression were remarkably decreased when restoration of miR-424 expression by specific mimics in cervical cancer cells. Further, we observed that miR-424 directly bound to Chk1 3′-UTR that contains a miR-424-binding site by dual-luciferase reporter assay. Thereby we confirm that Chk1 is a direct target of miR-424 in cervical cancer cells. In addition, it was firstly observed that phosphorylation and activation of Chk1 was regulated by miRNA though it has been known that Chk1 phosphorylation is subject to ATM and ATR control,[32,33] which may become a novel mechanism by which Chk1 and p-Chk1 are transformed to one another.

Chk1 has for long been known as a key player in determining cellular responses to DNA damage and governing G1/S, S and G2/M phase checkpoints.[34–36] Chk1 overexpression occurs in some types of tumors, such as colorectal cancer, breast cancer and epithelial ovarian cancer.[37–39] However, Chk1 was also not found to be overexpressed in some cancers including ovarian carcinoma, cervical cancer and breast cancer.[40] These discrepancies indicate further study to be needed. Here, we showed both Chk1 and p-Chk1 overexpressions in cervical cancer tissues compared with cervical normal tissues by IHC and western blot, and further observed that upregulation of both proteins was positively correlated to the increased progression and metastatic spread of the disease. To verify Chk1 effect on the progression of cervical cancer, we silenced Chk1 by specific RNAi in cervical cancer cells, and observed that single Chk1 siRNA-treated cervical cancer cells displayed significant reduction of Chk1 protein level and impaired activation of Chk1 at ser345, consequently resulting in restricted cell growth, enhanced apoptosis, blocked G1/S transition, and decreased motility and invasion capacity. Further experiments also revealed that Chk1 expression was positively correlated with cell metastasis-related protein MMP9 in cervical cancer tissues and silencing of Chk1 decreased MMP9 protein level in cervical cancer cells. Accordingly, we demonstrate that increased Chk1 through upregulating MMP9 plays a promoting role, as similar as downregulated miR-424, in the progression of cervical cancer.

Taken our findings together, miR-424 is downregulated in cervical cancer tissues and paralleled correlated with progression of the disease, and that, miR-424 regulates multiple cellular biological behaviors, such as retarding growth, inducing apoptosis, and reducing migration and invasion, by directly targeting Chk1, in cervical cancer cells. Overexpressions of Chk1 and p-Chk1 also exist in cervical cancer tissues and knockdown of Chk1 expression presents as similarly suppressive actions as mimic of miR-424 on cervical cancer cells. Thus, our findings suggest that

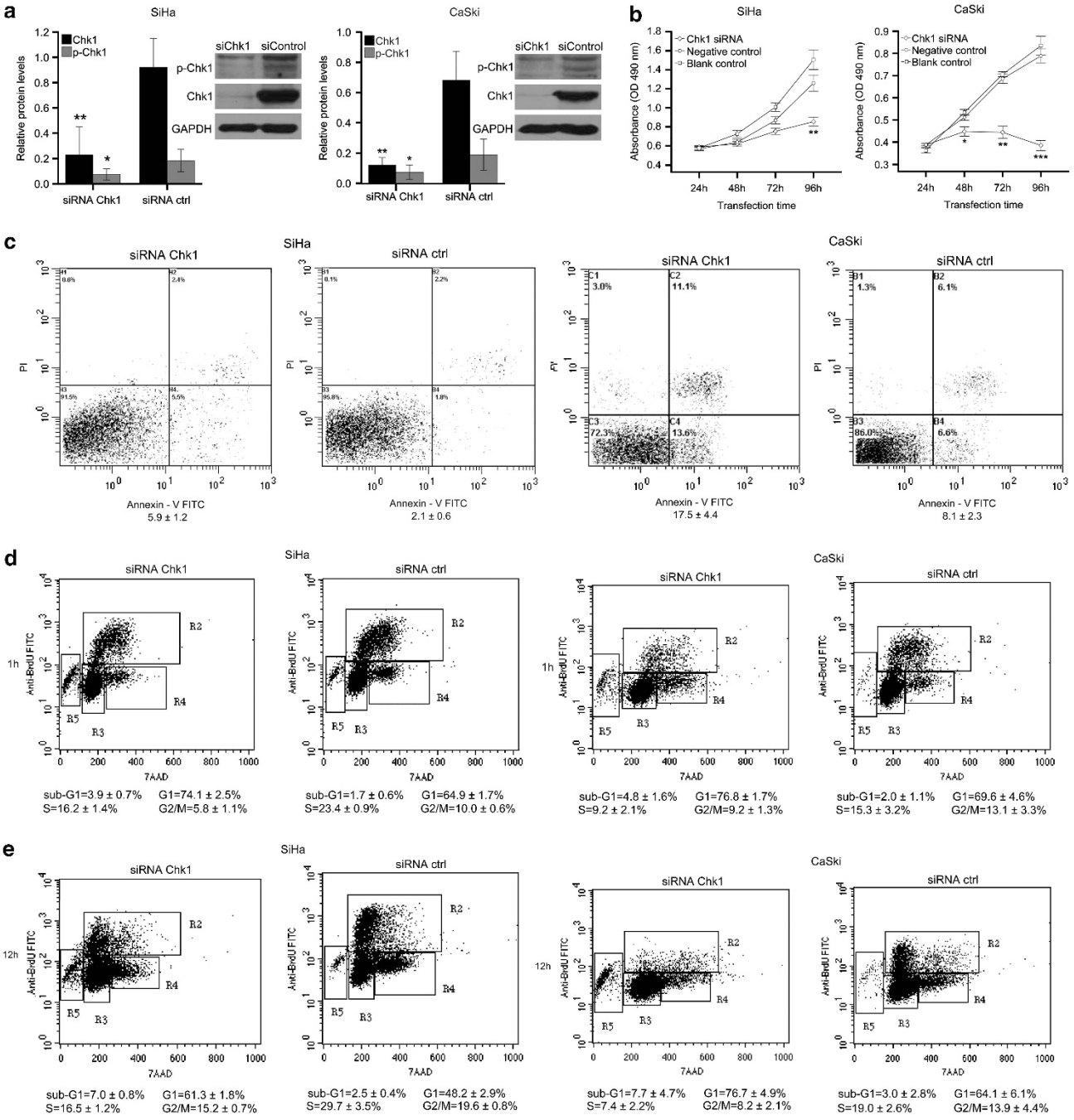

Figure 4. Specific siRNA targeting Chk1 suppressed both Chk1 and p-Chk1 protein expression in SiHa and CaSki cells and siRNA Chk1 inhibited proliferation, induced apoptosis and suppressed cell-cycle progression in cervical cancer cells. SiHa and CaSki cells were transfected with 50 nM of siRNA Chk1 or siRNA-negative control (siRNA ctrl) and subsequently assessed for transfection efficiency, cell proliferation, apoptosis and cell cycle. (**a**) Chk1 and p-Chk1 protein expression levels were determined by western blot analysis. GAPDH was served as the loading control. The relative expression levels were first normalized with that of GAPDH. Data were presented as mean ± s.d. from three independent experiments performed in duplicate. $*P<0.05$, $**P<0.01$ (**b**) Cells were seeded at 10 000 (SiHa) or 6000 (CaSki) cells per well into 96-well black plate. At the indicated time point after transfection, cell proliferation was assessed by the MTT assay. Data were presented as mean ± s.e. from three independent experiments, each performed in quintuplicate. $**P<0.01$ and $***P<0.001$. (**c**) 72 h after transfection, SiHa and CaSki cells were fixed, stained with Annexin-V FITC and PI, and analyzed by flow cytometry. Percentage of early apoptotic cells (%) was shown as mean ± s.d. below each grath. (**d**, **e**) Transfected cells were added by BrdU and incubated for 1 and 12 h, respectively. After stained by FITC anti-BrdU and 7-AAD, samples were measured by flow cytometry. Data were the mean of three independent experiments and presented as mean ± s.d.

downregulation of miR-424 contributes to the progression of cervical cancer at least partly via upregulation of target gene *Chk1* expression and phosphorylation of Chk1 protein. The miR-424 might become a potential predictor for prognosis and a candidate target for therapy for cervical cancer patients.

MATERIALS AND METHODS

Patients, cervical tissues and cell lines

A total of 147 primary cervical epithelial carcinoma and 74 cervical normal tissues were collected from September 2009 to December 2010 in Women's hospital, Zhejiang University School of Medicine, China.

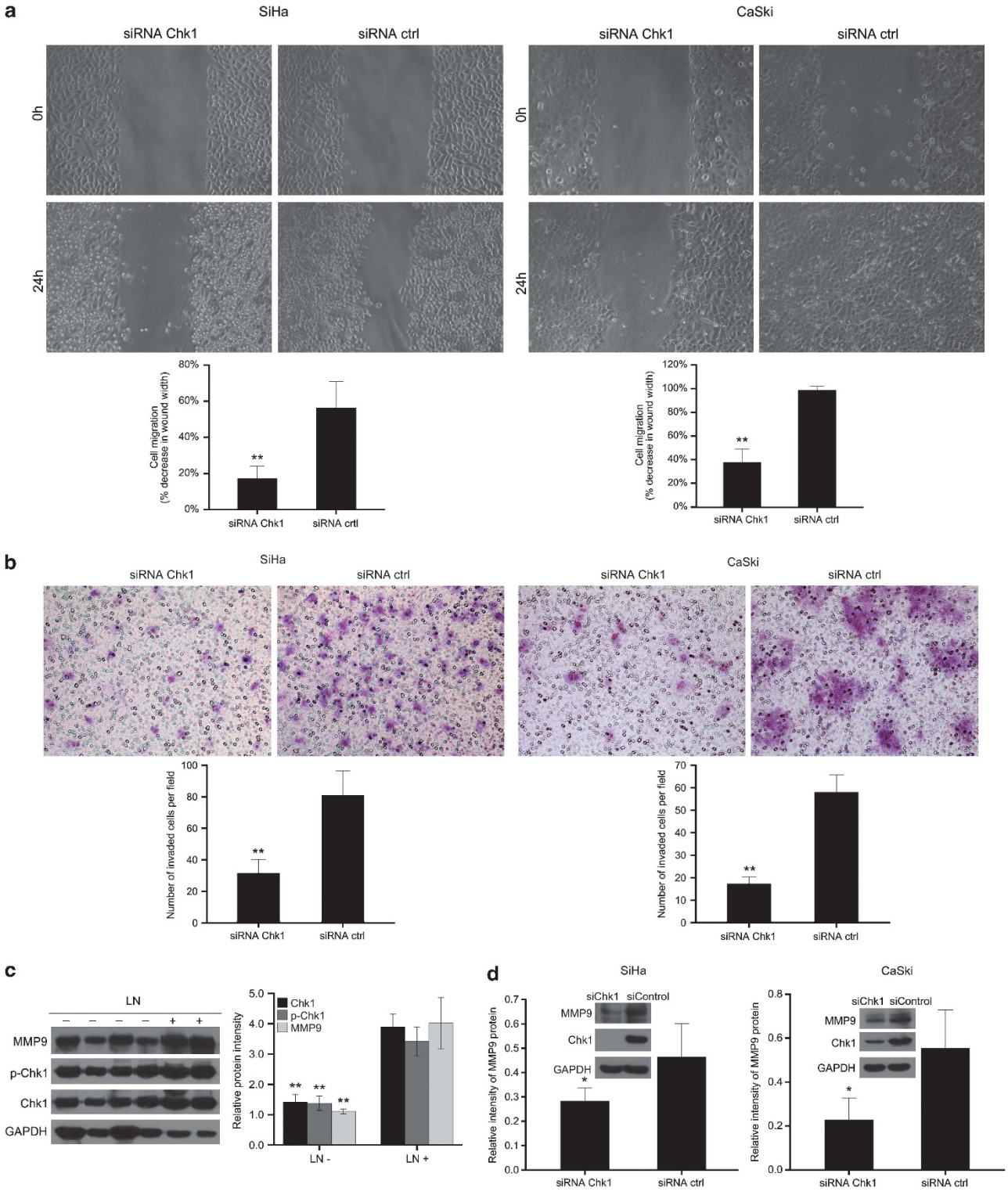

Figure 5. Inhibition of cell migration and invasion in SiHa and CaSki cells by siRNA Chk1 in an *in vitro* cell wound healing assay and matrigel chamber assay. Elevated protein levels of Chk1, p-Chk1 and MMP9 in cervical cancer tissues with lymph node metastasis, and suppressed MMP protein expression by siRNA Chk1. (**a**) Representative images of wound healing were taken on the day of the laceration (0 h) and day 1 (24 h) after the wound scratch. Data are presented as mean ± s.d. ($n = 3$). (**b**) The representation of invaded cells following knockdown of Chk1 using specific Chk1 targeted siRNA versus control (siRNA ctrl) in the SiHa and CaSki cell lines was shown by staining with crystal violet. Bars indicated the invasion activity compared with that of control ± s.d. $^{**}P < 0.01$. (**c**) Western blot validated the expression levels of Chk1, p-Chk1 and MMP9 protein in cervical cancer tissues with lymph node metastasis (LN +) and without (LN−). GAPDH was applied for normalization. Densitometric quantification of protein levels between LN− and LN + cervical cancer tissues were showed in the following bars. Mean ± s.e. $^{**}P < 0.01$. (**d**) 72 h after transfection with 50 nM of siRNA Chk1 or siRNA control, the protein level of MMP9 in SiHa and CaSki cells was detected by western blot. Bars indicated the relative protein levels that were normalized to GAPDH. Data were presented as mean ± s.d. ($n = 3$). $^{*}P < 0.05$.

All cervical cancer patients underwent radical hysterectomy and pelvic lymphadenectomy and their clinicopathological parameters, including age, clinical stage, pathological grade, SCC-Ag, tumor size, lymph node metastasis, vascular involvement and stromal invasion were collected and summarized in Table 1. Cervical normal tissues were collected from patients who underwent hysterectomy because of benign gynecological diseases. Specimens were obtained with informed consent and the study was approved by the Hospital Ethical Committee. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use. All the histological diagnoses for cervical carcinoma and normal tissues were reviewed and recognized by a senior pathologist.

Table 2. Positive correlation between Chk1 or p-Chk1 protein expression and MMP9 expression in cervical cancer tissues

	Chk1	*p-Chk1*
Pearson correlation		
Cervical cancer tissues		
MMP9		
Correlation coefficient	0.845[a]	0.842[a]
Sig. (two-tailed)	0.001	0.001
N	12	12

[a]Correlation is significant at the 0.01 level (two-tailed).

Two human cervical cancer cell lines, SiHa and CaSki, were purchased from the American Typed Cell Culture (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium with 10% FBS at 37 °C and 5% CO_2.

RNA extraction and qRT–PCR

Total RNA containing miRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa Otsu, Shiga, Japan).

To quantify the expression of mature miRNA, stem-loop RT–PCR was performed as described.[11] The product was further confirmed by TA cloning (TaKaRa) and sequencing (Invitrogen; Supplementary Figure S2). For normalization, U6 was used as an endogenous control. qRT–PCR analyses for mRNA of interest were performed using SYBR Premix Ex Taq (TaKaRa) as previously reported.[41] EEF1A1 was identified as a suitable internal control for human cervical tissue samples.[42] The primers used are shown in Supplementary Table S1.

Transfection

miR-424 mimic and nonspecific negative control (miRIDIAN miRNA Mimic Negative Control#1) were purchased from Dharmacon, Lafayette, CO, USA. siRNA against Chk1 and scrambled siRNA-negative control were synthesized by Ribobio, Guangzhou, China. For transient transfection, cell lines were seeded at 60% confluency, and transfection experiments were performed with miRNA mimics or Chk1 siRNA at a final concentration of 50 nM, respectively, using DharmaFECT 1 transfection reagent (Dharmacon)

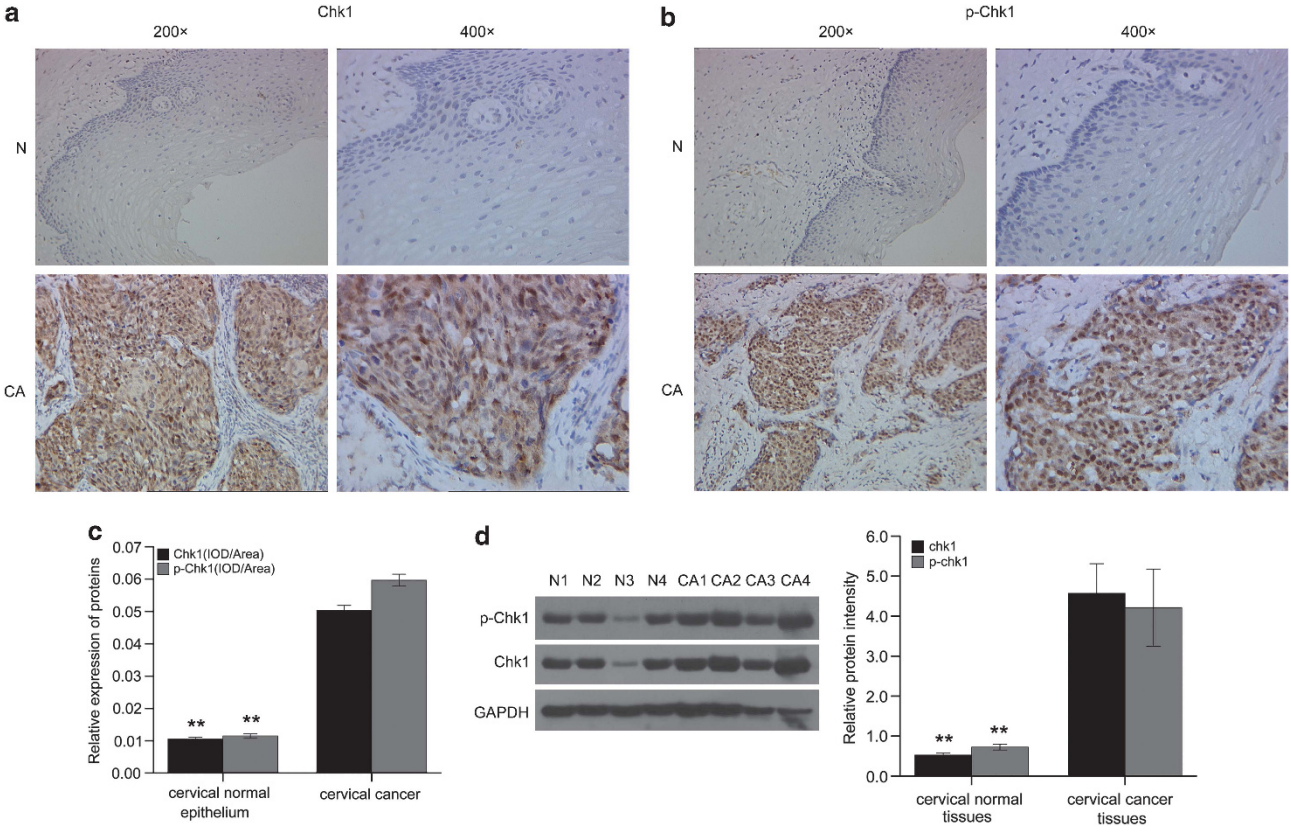

Figure 6. Protein levels of Chk1 and p-Chk1 are elevated in cervical cancer tissues either by IHC or western blot. (**a**, **b**) Expression of Chk1 (**a**) and p-Chk1 (**b**) in normal cervical epithelium and cervical tumor specimens were measured by IHC. The positive staining of both Chk1 and p-Chk1 was both cytoplasmic and, more strongly, nuclear. (**c**) The expression levels of Chk1 and p-Chk1 detected by IHC were determined by assessing its staining using software image pro-plus 6.0. The results were showed as integrated optical density (IOD)/area. Bar represented the average values of corresponding protein levels in normal cervical epithelium and cancer tumors ± s.e. (**d**) Western blot validated the expression levels of Chk1 and p-Chk1 protein in cervical cancer tissues (CA) compared with normal cervical tissues (N). The expression of GAPDH was applied for normalization. Densitometric quantification of protein levels between normal cervical tissues and cancer tissues was showed in the following bars. Mean ± s.e. $^{**}P < 0.01$.

Table 3. Negative correlation between miR-424 expression and Chk1 or p-Chk1 protein expression in cervical normal tissues and cervical cancer tissues, respectively

	miR-424	*Chk1*	*p-Chk1*
Pearson's correlation			
Cervical normal tissues			
miR-424			
Correlation coefficient	1	−0.740[a]	−0.754[a]
Sig.(two-tailed)		0.000	0.000
N	74	74	74
Chk1			
Correlation coefficient	−0.740[a]	1	0.766[a]
Sig.(two-tailed)	0.000		0.000
N	74	74	74
p-Chk1			
Correlation coefficient	−0.754[a]	0.766[a]	1
Sig.(two-tailed)	0.000	0.000	
N	74	74	74
Cervical cancer tissues			
miR-424			
Correlation Coefficient	1	−0.514[a]	−0.487[a]
Sig.(two-tailed)		0.000	0.000
N	147	147	147
Chk1			
Correlation coefficient	−0.514[a]	1	0.651[a]
Sig.(two-tailed)	0.000		0.000
N	147	147	147
p-Chk1			
Correlation coefficient	−0.487[a]	0.651[a]	1
Sig.(two-tailed)	0.000	0.000	
N	147	147	147

[a]Correlation is significant at the 0.01 level (two-tailed).

according to the manufacturer's guidelines. After overnight incubation, the culture medium was replaced with fresh Dulbecco's modified Eagle's medium containing 10% fetal bovine serum before further study. The transfection efficiency was detected by miRIDIAN miRNA mimic transfection control, a Dy547-labeled mimic based on *C. elegans* miRNA (cel-miR-67) for monitoring delivery into human cells (Supplementary Figure S3). The expression level of miR-424 in the transfected cells at 0, 24, 48, 72 and 96 h posttransfection was directly confirmed by qRT–PCR (Supplementary Figure S1).

Antibodies, western blot and IHC

Primary Chk1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (sc-8408) and Epitomics (Burlingame, CA, USA) (1740-1), primary p-Chk1 (ser345) antibodies were from Cell Signaling Technology (Danvers, MA, USA) (#2341) and Abcam (Cambridge, MA, USA) (ab47318), primary MMP9 was from Epitomics (2551-1), and GAPDH antibody was from Santa Cruz (sc-47724). Secondary antibodies used for western blot were purchased from Dawen Biotec (Zhejiang, China) and those used for IHC were from Dako (Dako Diagnostica, Hamburg, Germany).

For western blot, transfected cells and tissue samples were harvested and homogenized with lysis buffer. A total of 50 μg protein was separated by denaturing 8% SDS–polyacrylamide gel electrophoresis. Western analysis was conducted as described.[43] Protein levels were normalized to GAPDH and fold changes were determined.

For IHC staining, the expression of Chk1 and p-Chk1 protein was detected in 221 cases of cervical tissues that were simultaneously used to examine the expression level of miR-424. Tissue sections (4 μm) were cut from FFPE blocks and IHC staining was performed using the Envision method.[44] In brief, IHC scoring analyses of Chk1 and p-Chk1 expression were evaluated by Image pro-plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

Luciferase reporter assay

For luciferase reporter experiments, a wild-type Chk1 3′-UTR segment of 133 bp was amplified by PCR from human cDNA library, confirmed by sequencing (Invitrogen; Supplementary Figure S4), and cloned into the XbaI and Sac I site of pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA). Mutant Chk1 3′-UTR was generated based on the pmirGLO-Chk1-3′-UTR by mutating three nt that is recognized by miR-424, confirmed by sequencing (Supplementary Figure S5). Cotransfection of miRNA mimic and the reporter gene were performed using DharmaFECT duo transfection reagent (Dharmacon) following the manufacturer's manual. Briefly, assayed cells were grown to about 60% confluence in a 96-well tissue culture plate, cells were cotransfected with 0.2 μg/ml pmirGLO-Chk1 or mutant pmirGLO-Chk1, and with 50 nM of tested miRNAs. After 48 h, the luciferase activities were assessed using the Dual-Glo Luciferase Assay System (Promega) by measuring the intensity of chemiluminescence in a luminometer (Thermo Fisher Scientific, Waltham, MA, USA). The experiments were performed in triplicate and repeated at least three times with negative controls. The primers of Chk1 3′-UTR and Chk1 3′-UTR-mut are shown in Supplementary Table S1.

Cell proliferation analysis

To evaluate cell proliferation, SiHa (1×10^4 cells per well) and CaSki cells (6×10^3 cells per well) were separately plated in 96-well plates and transfected on the following day with 50 nM of miRNA mimic or siRNA. At 24, 48, 72 and 96 h after transfection, cell viability was identified using 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. A 20-μl of MTT solution was added to each well and continued incubation for 4 h. Then, the supernatant was removed and 150 μl of dimethylsulfoxide (DMSO) was added to stop the reaction. Finally, the optical density was determined with a microplate spectrophotometer (ELx800, Bio-TEK, Winooski, VT, USA) at a wavelength of 490 nm. And the proliferation curve was generated based on absorbance and time.

Apoptosis and cell-cycle assay

For apoptosis assays, 72 h posttransfection, adherent cells were harvested and washed twice with phosphate-buffered saline. Annexin-V in combination with propidium iodide (Biouniquer) was added to the cells and samples were analyzed within 30 min after staining. Quantificate of fluorescence was analyzed by flow cytometry (Beckman Coulter, Fullerton, CA, USA).

For cell-cycle analysis, 48 h after transfection, 10 μl of BrdU solution (1 mM) was directly added to each ml of culture media. The treated cells were then incubated for the desired length of time (1 and 12 h, respectively), fixed and stored overnight before staining. The following staining protocol was done according to the manufacturer's guidelines (BD Pharmingen BrdU Flow Kit, BD Biosciences, San Jose, CA, USA). Finally, samples were analyzed by flow cytometry (Becton Dickinson, FACSCalibur, Franklin Lakes, NJ, USA).

Cell migration and invasion

Cell migration potential was evaluated using a wound-healing assay. Cells were seeded in 12-well plates and cultured for 24 h to form confluent monolayers. A wound was created by dragging a 10-μl pipette tip through the monolayer and the plates were washed with phosphate-buffered saline to remove cellular debris. Photograph wound images when the scrape wound was introduced (0 h) and at a designated interval (24 h) after wounding using an inverted microscope. The wound gaps at each time point were measured and the speed of migration was acquired by dividing the length of the gap by the culture time. Two replicates in each of three independent experiments were performed.

To measure cell invasion, cells of posttransfection were harvested and 1×10^5 cells were seeded atop inserts membranes coated with matrigel (BD Biosciences) in 24-well plate with 8.0 μm pores (Corning). Cells were incubated with serum-free medium and translocated towards medium with 10% FBS for 24 h. The cells remained in the upper compartment were removed by cotton swabs, and those who had invaded through the matrix were stained by cell stain 0.1% crystal violet and counted under a light

Table 4. Association between Chk1 or p-Chk1 protein expression and clinicopathological parameters of cervical cancer patients

Characteristic	*Total (n = 147)*	*Chk1 expression*		P	*p-Chk1 expression*		P
		Low	*High*		*Low*	*High*	
Age, years				0.868			0.306
⩽35	17	13	4		11	6	
>35	130	97	33		99	31	
FIGO stage				**0.002**			**0.026**
IB	108	88	20		86	22	
IIA	39	22	17		24	15	
Pathological grade				0.422			0.422
Grade 1	3	3	0		3	0	
Grade 2	131	99	32		99	32	
Grade 3	13	8	5		8	5	
SCC-Ag, ng/ml				0.062			0.49
<4	128	99	29		97	31	
⩾4	19	11	8		13	6	
Tumor size, cm				0.645			0.202
<4	119	90	29		96	33	
⩾4	28	20	8		24	4	
Lymph nodes metastasis				**3.00E-07**			**4.00E-05**
Negative	123	102	21		100	23	
Positive	24	8	16		10	14	
Vascular involvement				0.06			**3.00E-06**
Negative	98	78	20		85	13	
Positive	49	32	17		25	24	
Deep stromal invasion				**0.027**			**0.009**
<66%	90	73	17		74	16	
⩾66%	57	37	20		36	21	

Abbreviations: Ag, antigen; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma.
Bold values were presented as $P<0.05$.

microscope with five individual fields per insert. Results are presented as an average of triplicate experiments.

Statistical analysis

Statistics were assessed using software packages SPSS version 16.0(WPSS Ltd, Surrey, UK). The results were presented as mean ± s.e./s.d. Relative quantification of miRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method. The differences in miRNA or protein expression between cervical cancer and normal cervical tissues were evaluated using the nonparametric Mann–Whitney *U*-test. Correlations between miRNA or protein expression and the clinicopathological parameters were analyzed by χ^2-test and Fisher's exact probability test. For experiments *in vitro*, student's *t*-test was used to analyze the differences between two groups. All *P*-values were two sided and *P*-values <0.05 were regarded as significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

The authors declare that the material is an original research, has not been previously published and has not been submitted for publication elsewhere while under consideration.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)