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Elevated Expression Levels of Long Non-Coding RNA, Loc554202, Are Predictive of Poor Prognosis in Cervical Cancer

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Cervical cancer remains one of the most common causes of gynecological cancer-associated death. Long non-coding RNA Loc554202 (lncRNA Loc554202) has been reported to be involved in the development of several types of cancer. However, the role of Loc554202 in cervical cancer remains unclear. In this study, we measured the expression levels of Loc554202 in cervical cancer tissues from 120 patients. The quantitative real-time PCR analysis showed the expression levels of Loc554202 were significantly higher in cervical cancer tissues compared with the adjacent non-tumor tissues. Elevated expression levels of Loc554202 were significantly associated with tumor size ($p = 0.006$), FIGO stage ($p = 0.015$), HPV ($p = 0.001$), and lymph node metastasis ($p = 0.002$). Kaplan-Meier analysis clearly illustrated that patients with high expression levels of Loc554202 had a lower overall survival rate compared to patients with lower expression ($p = 0.0013$). Furthermore, we show that Loc554202 is an independent poor prognostic factor through multivariate analysis. Subsequently, using cervical cancer cell lines, HeLa and ME-180, we decreased the expression levels of Loc554202 with siRNA. As results, the proliferation ability of cervical cancer cells was inhibited and apoptosis was induced after Loc554202 knockdown, as judged by viability assay, colony formation, and flow cytometry. Moreover, knockdown of Loc554202 expression down-regulated Bcl-2 expression and conversely up-regulated Bax expression in cervical cancer cells using Western blotting analysis. In conclusion, elevated levels of Loc554202 are predictive of poor prognosis in cervical cancer. We suggest that Loc554202 may serve as a potential therapeutic target for cervical cancer.

Keywords: cervical cancer; long non-coding RNA; Loc554202; prognosis; proliferation
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Introduction

Cervical cancer remains one of the most common causes of gynecological cancer-associated death, with an estimated approximately 528,000 new cases and 266,000 deaths annually (Patch et al. 2015; Cancer Genome Atlas Research Network 2017). The prevalence of cervical cancer is high in women infected with human papillomavirus (HPV), especially HPV 16 and 18, accounting for 60-70% of cervical cancer around the world (Khan et al. 2005). Surgical resection, radiotherapy and platinum-based chemotherapy (involving either cisplatin or carboplatin) are still considered as major nursing care plans (Rose et al. 1999). However, recurrent episodes of cervical cancer usually occur, although effective for early-stage patients. Hence, exploring the diagnostic and prognostic potential of specific and sensitive biomarkers in cervical cancer is urgently needed.

The human genome encodes many long noncoding RNAs (lncRNAs), which are different from other structural RNAs, including tRNAs, rRNAs, and snRNAs (Tuck and Tollervey 2013). Most lncRNAs are likely to be transcribed and probably have functional roles in various biological processes (Braconi et al. 2011). Increasing evidence has established the potential relation between dysregulation of lncRNA expression and numerous human diseases, such as cancer, metabolic disease, neurodegenerative, psychiatric disease, and immune dysfunction (Harries 2012). In fact, a number of lncRNAs are considered to be closely related to initiation and progression of cancer (Prensner and Chinnaiyan 2011; Prensner et al. 2013). The lncRNA NEAT1 regulated by estrogen receptor α has been shown to stimulate tumorigenenicity in prostate cancer (Chakravarty et al. 2014). LncRNA GAS5 behaves as a suppressor of stomach cancer, and its knockdown promoted turnover of YBX1 protein, which then down-regulated YBX1-

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transactivated p21 expression and attenuates cell cycle at G1 stage (Liu et al. 2015). Besides playing pivotal roles in occurrence and development of cancer, several lncRNAs are deemed as promising biomarkers to reflect the prognosis and outcome of cancer (Malik et al. 2014). For instance, MALAT1 expression is increased in colorectal cancer and pancreatic duct adenocarcinoma tissues, having been predicated as a biomarker for prognosis and metastasis in these diseases (Zheng et al. 2014). High expression of lncRNA HOTAIR is strongly associated with clinical characteristics in cervical cancer including FIGO stage, lymph node metastasis, invasion depth, and tumor size (Huang et al. 2014). Pancreatic cancer patients of the high-lncRNA-PVT1 group had obviously shorter overall survival times than those of the low-expression group (Huang et al. 2015).

The gene for lncRNA Loc554202 is located on human chromosome 9p21.3. Loc554202 is the host gene of miR-31, and miR-31 is transcribed from within the first intron of Loc554202 on human chromosome 9 (Corcoran et al. 2009). Augoff et al. (2012) also found the epigenetic regulation between Loc554202 and miR-31 by identifying a major CpG island upstream of miR-31 locus. Recent reports have demonstrated the involvement of Loc554202 in a variety of human cancers including breast cancer (Shi et al. 2014), colorectal cancer (Yang et al. 2016), and bladder cancer (Liu et al. 2016). In breast cancer, knockdown of Loc554202 decreased cell proliferation, induced apoptosis and inhibits migration/invasion in vitro and impeded tumorigenesis in vivo. By contrast, Loc554202 appeared to have lower expression in the colorectal cancer tissues (Ding et al. 2015). These results further enhanced our research interests for investigating the potential biological and clinical significance of Loc554202 in cervical cancer.

In the current study, the expression of Loc554202 was investigated in cervical cancer, and the clinicopathological characteristics and prognostic value of Loc554202 were assessed. Then, knockdown of Loc554202 mediated by siRNA was used to determine its relevance to biological behavior in cervical cancer. Our study may provide a good diagnostic and prognostic marker in cervical cancer.

Materials and Methods

Patients and clinical samples

A total of 120 cervical cancer tissues and the paired adjacent non-tumor cervical tissues, between October 2015 and September 2016, were collected from patients undergoing hysterectomy without radiotherapy or chemotherapy at the Department of Obstetrics & Gynecology, Shanghai Jiao Tong University Affiliated Renji Hospital. The diagnosis was evaluated by two independent pathologists and clinicopathological characteristics were retrieved. After collection, all fresh specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Clinical follow-up information was obtained by telephone or the outpatient records. Written informed consent was obtained from all of the patients. This study was approved by the Research Ethics Committee of Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital.

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from the tissues and cells by Trizol reagent (Thermo Fisher Scientifc, Waltham, MA, USA) according to the manufacturer's protocols and then converted to cDNA using a reverse transcription kit (Takara). Real-time PCR and data collection were performed using Applied Biosystems 7500 Sequence Detection system. The following primer sets for Loc554202 and GAPDH were used: Loc554202 forward: 5′-TCTCTGGTGCTTCCCTCCTT-3′, reverse: 5′-GATCTAAGCTTGAGCCCCCA-3′; GAPDH forward: 5′-AGAGGCAGGGATGATGTTCTG-3′, reverse: 5′-GACTCATGA CCACAGTCCATGC-3′. The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ methods.

Cell culture and small interfering RNA (siRNA) transfection

The human cervical cancer cell lines, HeLa and ME-180, were purchased from the Cell Bank of Type Culture of the Chinese Academy of Sciences (Shanghai, China). They were cultured in RPMI-1640 medium (Gibco, ThermoScientific, MA) containing 10% fetal bovine serum (FBS, Gibco). All the cells were maintained in a humidified incubator with 5% CO_2 at 37°C. Three siRNA specifically targeting Loc554202 (si-Loc554202) and a scrambled nucleotide (si-NC) were purchased from Life Technologies (Carlsbad, California, USA). The target sequences of si-Loc554202 are as follows: si-Loc554202-1: CCUGGUUGAGCUGAGGUCUUCAUAG, si-Loc554202-2: GGAGCGCUUUGUGUGAGAAGUUGAA, si-Loc554202-3: GCAGGUAGAGAUGGAUUCCUGGAAA. For cell transfection, the cells cultured in growth media to grow to half confluence (2×10^5 cells/well) prior to transfection with corresponding siRNA using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for further experiments.

Cell viability assay

Cell viability was assessed using the cell counting kit-8 (CCK-8) assay. In brief, cells transfected with si-Loc554202 or si-NC were seeded in 96-well plates at 2×10^3 cells/well. Then each well was added 10 μL CCK-8 solution at time points of 0, 24, 48, 72 and 96 h, respectively. Following incubation for 2 h at 37°C, the optical density value was measured at 450 nm using a microplate reader.

Colony formation assay

Transfected cells were seeded into 6-well plates at a density of 200 cells per well and incubated for 4 days. Then cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were stained with crystal violet and the numbers of single colonies (containing > 50 cells) were counted using a light microscope.

Flow cytometry for cell apoptosis analysis

Cell apoptosis were evaluated using flow cytometry analysis of cells expressing si-Loc554202. Briefly, transfected cells were seeded on 6-cm dishes at a density of 1×10^5 cells/dish and collected until 80% confluence. After fixed with suspension in 70% ethanol for 30 min at 4°C, apoptosis was detected by Annexin V-APC/7-AAD apoptosis detection kit from KeyGen Biotech (Nanjing, China) according to the manufacturer's instructions.

Western blot assay

Total protein was obtained from transfected cells using RIPA

lysis buffer (Beyotime, Shanghai, China) supplemented with 1% protease inhibitor cocktail (Roche). Equal quantities of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene fluoride membrane (Sigma, St. Louis, MO, USA). The membrane was blocked with 5% skimmed milk at room temperature for 2 h, and then incubated with overnight at 4°C with primary antibodies against Bcl-2, Bax and GAPDH (dilution 1:1,000; Cell Signaling Technology, Danvers, MA). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000, #2534, Cell Signaling Technology, MA) at room temperature for 2 h, the protein bands were visualized using super ECL detection reagent (Pierce, Rockford, IL, USA) according to the manufacture's instruction.

Statistical analysis

All statistical analyses were conducted using SPSS 19.0 statistical software package (IBM, Armonk, NY, USA). All quantitative data from three independent experiments were expressed as mean ± standard deviation (SD) and analyzed using independent-sample t-test. All categorical data was analyzed by the chi-square test. According to the median value of Loc554202 expression levels, total 120 cervical cancer patients were divided into a high-expression group (n = 66) and a low-expression group (n = 54). Survival analysis was performed by Kaplan-Meier method with the log-rank test. The significance of various variables for survival was analyzed using univariate and multivariate Cox regression methods. A p-value of less than 0.05 was considered to represent statistically significant.

Results

Loc554202 expression levels are significantly higher in cervical cancer

To characterize the role of Loc554202 in cervical cancer, qRT-PCR was firstly performed in 120 cervical cancer tissues with paired adjacent non-tumor tissues. As shown in Fig. 1, the expression levels of Loc554202 were significantly higher in cervical cancer tissues (1.324 ± 0.216) compared with normal tissues (0.723 ± 0.342), raising the possibility that Loc554202 is a potential biomarker for cervical cancer. We further divided 120 cervical cancer patients to a high-expression group (n = 66) and a low-expression group (n = 54) according to the median value of Loc554202 expression levels. The association between Loc554202 and the clinicopathological outcomes is shown in Table 1; Loc554202 expression was significantly associated with tumor size (p = 0.006), FIGO stage (p = 0.015), HPV (p = 0.001) and lymph node metastasis (p = 0.002). There was no significant correlation between Loc554202 expression and age and pathological differentiation.

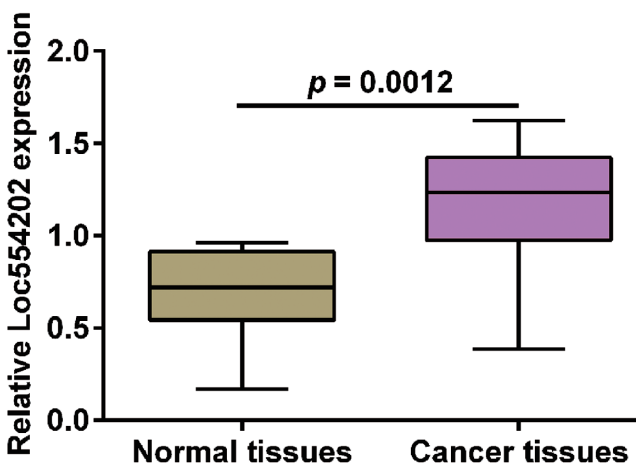

Fig. 1. The relative expression of Loc554202 in cervical cancer tissues.
The expression levels of Loc554202 were significantly higher in cervical cancer tissues than those in adjacent non-cancer tissues (Normal tissues).

Elevated levels of Loc554202 are predictive of poor prognosis in patients with cervical cancer

To investigate whether the higher levels of Loc554202 affected the prognosis of cervical cancer patients, we performed the Kaplan–Meier analysis with log-rank test. As shown in Fig. 2, the median follow-up time for overall survival was 43 months for all patients. Patients with low Loc554202 expression (5-year overall rate, 37.8%) had higher overall survival rate than those with high Loc554202 expression (5-year overall rate, 21.3%) (p = 0.0013). In addition, univariate analysis indicated that overall survival was significantly correlated with tumor size, FIGO stage, lymph node metastasis and Loc554202 expression levels (Table 2). Moreover, multivariate Cox proportional hazard model was used to evaluate the effects of the independent factors on overall survival. As depicted in Table 2, Loc554202 expression (HR = 2.875, 95% CI: 1.539-3.536; p = 0.007), tumor size (HR = 1.654, 95% CI: 1.273-2.147; p = 0.013) and FIGO stage (HR = 2.369, 95% CI: 1.542-3.459; p = 0.032) were recognized as independent prognostic factors on overall survival in cervical cancer patients. These results strengthen our conjecture that Loc554202 expression may have a significant correlation with poor prognosis in cervical cancer.

Loc554202 knockdown suppresses proliferation of cervical cancer cells

Loc554202 was overexpressed in cervical cancer, prompting us to explore its biological function upon selective inhibition. Total three siRNAs were designed and transfected into cervical cancer cell lines, HeLa and ME-180 to knock down the Loc55420 expression. Using qRT-PCR analysis, we found si-Loc554202-1 had the highest knockdown efficiency, and the expression level was inhibited up to 86.6%, which was obviously higher than si-Loc554202-2 or si-Loc554202-3 (Fig. 3A, $p < 0.01$, $p < 0.05$). Thus, si-Loc554202-1 was selected for subsequent experiments. As shown in Fig. 3B, CCK-8 assay indicated that transfection of both HeLa and ME-180 with si-Loc554202-1 significantly decreased cell viability by 65.32% and 68.96%, respectively ($p < 0.001$). Consistent with these results, colony formation assay showed that clonogenic survival decreased when cervical cancer cells were transfected with si-Loc554202-1 compared with si-NC (Fig.

Table 1. Association between the Loc554202 expression and clinicopathological parameters in patients with cervical cancer.

Variables	Cases (n = 120)	Loc554202 expression		p Value (chi-square test)
		Low (n = 54)	High (n = 66)	
Age				0.458
≤ 50	43	17	26	
> 50	77	37	40	
Tumor size(cm)				**0.006**
< 4.0	86	45	41	
≥ 4.0	34	9	25	
FIGO stage				**0.015**
Ib-IIa	71	38	33	
IIb-IIIa	49	16	33	
HPV				**0.001**
(−)	67	39	28	
(+)	53	15	38	
Lymph node metastasis				**0.002**
Negative	56	35	21	
Positive	64	19	45	
Pathological differentiation				0.068
Well or moderately	87	43	44	
poorly	33	11	22	

FIGO, international federation of gynecology and obstetrics; HPV, human papilloma virus.

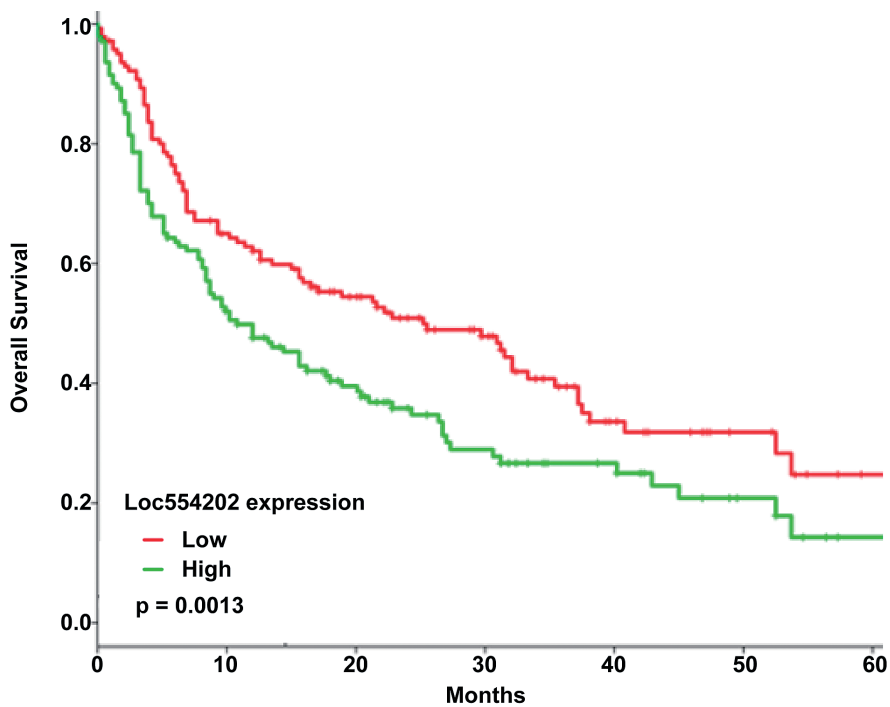

Fig. 2. Kaplan-Meier curves for overall survival.
Kaplan-Meier curves for overall survival based on Loc554202 expression in group of 120 cervical cancer patients, including a high-expression group (n = 66) and a low-expression group (n = 54) ($p < 0.05$, log-rank test).

3C, $p < 0.001$). Taken together, Loc554202 might have a proliferation promoting function in cervical cancer cells.

Loc554202 knockdown induces cervical cancer cell apoptosis

To examine the mechanism underlying the inhibition of cell proliferation, the cell apoptosis was detected by flow

Table 2. Univariate and multivariate analyses for prognostic parameters influencing overall survival of cervical cancer patients by Cox regression analysis.

Variables	HR	95% CI	p value
Univariate analysis			
Age	0.938	0.736-1.268	0.557
Tumor size (cm)	1.334	0.987-1.756	**0.003**
FIGO stage	1.310	0.968-2.635	**0.034**
HPV	2.165	1.534-2.942	0.142
Lymph node metastasis	2.546	1.347-3.241	**0.036**
Pathological differentiation	1.865	1.334-2.342	0.352
Loc554202 expression	3.435	2.423-3.857	**0.003**
Multivariate analysis			
Tumor size (cm)	1.654	1.273-2.147	**0.013**
FIGO stage	2.369	1.542-3.459	**0.032**
Lymph node metastasis	1.914	1.845-2.596	0.124
Loc554202 expression	2.875	1.539-3.536	**0.007**

FIGO, international federation of gynecology and obstetrics; HPV, human papilloma virus; HR, hazard ratio; CI, confidence interval.

cytometry in cervical cancer cells after Loc554202 knockdown. As shown in Fig. 4A, Annexin V-APC *vs.* 7-AAD plots from the gated cells represented the populations corresponding early (Annexin V+/7-AAD−) and late (Annexin V+/7-AAD+) apoptotic cells in HeLa and ME-180 cells after si-Loc554202-1 or si-NC transfection. Further statistical analysis (Fig. 4B) indicated that a significantly elevated percentage of early and late apoptotic cells after Loc554202 knockdown in both HeLa cells ($p < 0.001$, $p < 0.05$) and ME-180 cells ($p < 0.01$, $p < 0.001$).

Furthermore, several molecules associated with apoptotic regulation were determined by Western blot analysis (Fig. 4C). The level of anti-apoptotic protein Bcl-2 was obviously decreased upon Loc554202 knockdown. Consistently, we observed a significant increase in Bax expression. These results suggest that knockdown of Loc554202 inhibited cervical cancer cell proliferation partially through activating apoptotic pathway.

Discussion

Nowadays, the knowledge about the etiology and pathogenesis of cervical cancer is increasing rapidly. lncRNAs constitute a heterogeneous group of RNA molecules in eukaryotic cells and play a role in a wide variety of biological processes (Hanahan and Weinberg 2000). Previous studies have shown that expression of several lncRNAs is dysregulated in cancer tissues, such as MEG3 (Luo et al. 2015), CCHE1 (Yang et al. 2015), and HOTAIR (Ding et al. 2015). In the present study, high levels of Loc554202 were observed in cervical cancer tissues compared with the surrounding non-tumor cervical tissues. According to previous findings, different cancer types may have different trend to the expression of Loc554202 compared with their match normal tissues. Shi et al. (2014) and Liu et al. (2016) found that low Loc554202 expression was observed in normal breast and bladder tissues, while the expression in breast and bladder cancer appears to be elevated. Conversely, the levels of Loc554202 mRNA were lower in colorectal cancerous tissues relative to adjacent normal samples (Ding et al. 2015).

Accordingly, we speculate that Loc554202 may function as an oncogene in cervical carcinogenesis and play a key role in cancer clinicopathological phenotypes. Unexpectedly, the expression of Loc554202 was associated with the tumor size ($p = 0.006$), FIGO stage ($p = 0.015$), HPV ($p = 0.001$), and lymph node metastasis ($p = 0.002$). A strong relationship between high level of Loc554202 and cervical cancer growth has led us to hypothesis that Loc554202 may lead to poor outcome in cervical cancer patients. Kaplan–Meier analysis showed that patients with high Loc554202 expression had shorter overall survival rate than those with low Loc554202 expression. Yang et al. (2016) reported that down-regulation of Loc554202 is related with histological grade, lymph nodes metastasis, and TNM stage in colorectal cancer: 57 of 81 cancer tissues with poorly histological grade, 35 of 51 cancer tissues with

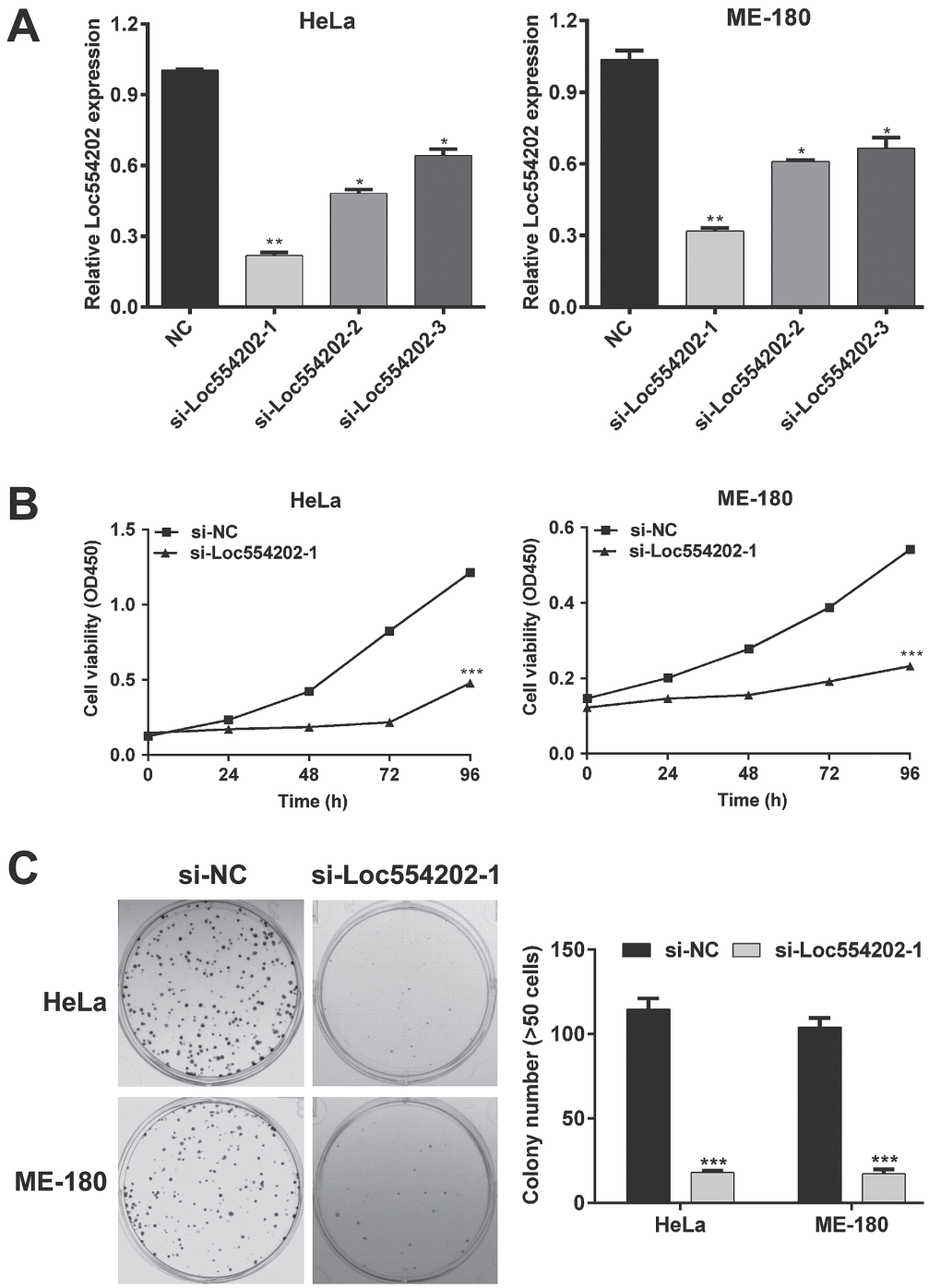

Fig. 3. Knockdown of Loc554202 expression inhibits cell proliferation ability of cervical cancer cells.
(A) Loc554202 expression levels were determined by quantitative RT-PCR in HeLa and ME-180 cells following transfection with si-Loc554202 (-1, 2, 3) or si-NC. (B) The CCK-8 assay was used to determine the cell viability in HeLa and ME-180 cells following transfection with si-Loc554202-1 or si-NC. (C) A colony formation assay was performed on HeLa and ME-180 cells transfected with si-Loc554202-1 or si-NC. All experiments were performed three times. Data were expressed as mean ± standard deviation (SD) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. si-NC).

positive lymph nodes metastasis, and 53 of 83 cancer tissues with TNM stage III-IV. Shi et al. (2014) showed that Loc554202 expression is linked with advanced pathologic stage and tumor size. Based on high-level expression profiles of Loc554202 in breast cancer and cervical cancer samples, the role of Loc554202 in cervical cancer is similar to that in the breast cancer. In addition, the cervical cancer patients with high-expression of Loc554202 had a poor outcome, which is distinct from colorectal cancer. Besides, multivariate analysis showed that Loc554202 is a strong independent predictor of overall survival in patients with cervical cancer, indicating that Loc554202 might function

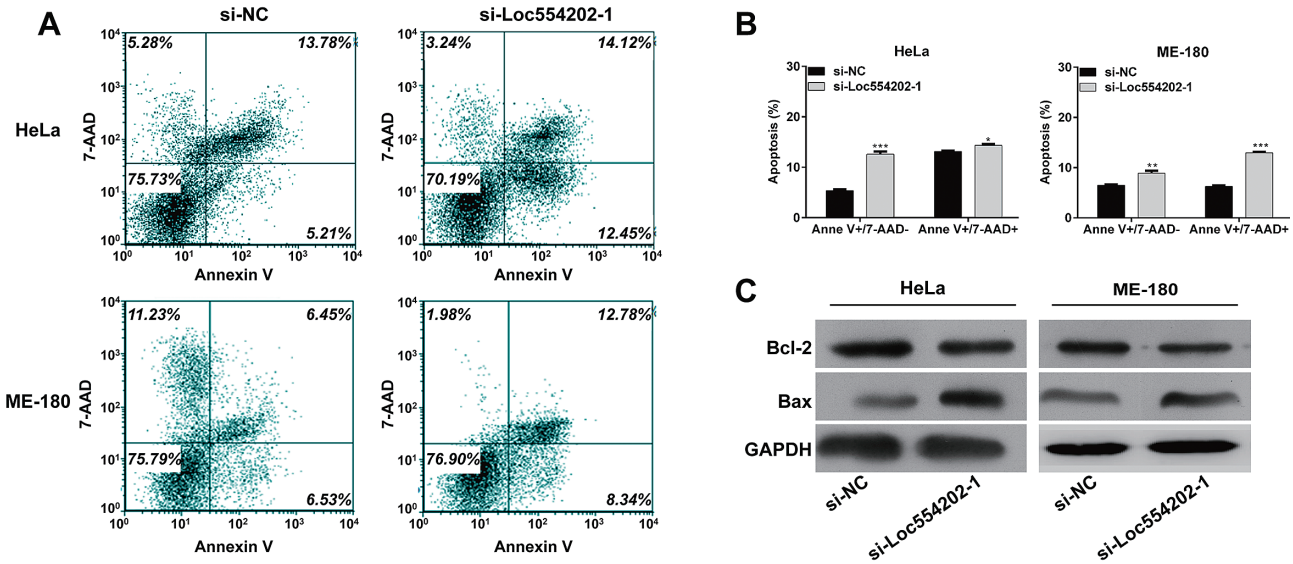

Fig. 4. Knockdown of Loc554202 induces apoptosis in cervical cancer cells.
(A) Cell apoptosis was analyzed by Annexin V/7-AAD staining and flow cytometry in HeLa and ME-180 cells after transfection with si-Loc554202-1 or si-NC. (B) The early apoptosis and late apoptosis were calculated in HeLa and ME-180 cells following transfection with si-Loc554202-1 or sh-NC. (C) The expression levels of Bcl-2 and Bax were determined by Western blotting analysis in HeLa and ME-180 cells transfected with si-Loc554202-1 or sh-NC. All experiments were performed three times. Data were expressed as mean ± standard deviation (SD) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. si-NC).

as an indicator for prognosis for cervical cancer.

Malignant tumors are characterized by uncontrolled proliferation and invasion (Evan and Vousden 2001; Malumbres and Carnero 2003). In this study, the biological effects of Loc554202 in Hela and ME-180 cells were investigated. As a result, knockdown of Loc554202 suppressed cell proliferative activity and colony formative capability of the two cervical cancer cell lines, which indicated that Loc554202 is implicate as an oncogene in cervical cancer. Several lncRNAs modulate cancer cells survival though interfering with pro-and anti-apoptotic molecules. Bcl-2, an integral membrane protein, locates on the mitochondria-out membrane and has anti-apoptotic property (Yang et al. 1997). When stimulated by apoptotic signals, cytochrome c is quickly released into the cytosol from mitochondria, then binds to Apaf-1 and activates caspase-like protease to bring about fast and irreversible apoptosis (Slee et al. 1999). Interestingly, the release of cytochrome c from mitochondria can be blocked by Bcl-2 but promoted by the proatoptotic family members Bax (Kluck et al. 1997; Lindsten et al. 2000). The decreased susceptibility of cells to death stimuli is associated with high ratio of Bcl-2 and Bax, while increased by low Bcl-2/Bax ratio (Osorio et al. 1997). In the present study, a significant increase in the percentage of early and late apoptotic cells was observed in Hela and ME-180 cells after Loc554202 knockdown.

Moreover, Bax upregulation and Bcl-2 downregulation were found in Hela and ME-80 cells depleted of Loc554202. In this light, we assumed that Loc554202 deficiency accelerated cervical cancer cells from apoptosis is associated with an attenuated ratio of Bcl-2 to Bax. Additionally, Liu et al. (2016) have found that Loc554202 protects bladder cancer cells from apoptosis and that down-regulation of Bcl-2 and upregulation of caspase-3 and caspase-9 were detected in Loc554202-knockdown bladder cancer cells, indicating activation of mitochondria apoptosis pathway. We supposed that the apoptotic effect of Loc554202 in cervical cancer may be mediated via the mitochondrial pathway.

In conclusion, we demonstrate that Loc554202 expression is increased in cervical cancer tissues and its high expression is predictive of poor prognosis in patients with cervical cancer. Our results provide new insights regarding the role of Loc554202 in cervical cancer development and have clinical significance for the exploration of lncRNA-based cancer therapy.

Conflict of Interest

The authors declare no conflict of interest.

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