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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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 predicted 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 Scientific, 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′-TCTCTGTGTCTTTCCCTCCTT-3′, reverse: 5′-GTATCTAAGCTTGAAGCCCCCA-3′; GAPDH forward: 5′-AGAGGCAGGGGATGATGTTCTG-3′, reverse: 5′-GACTCATGCAACAGTCCATGC-3′. The relative quantitative value was expressed by the 2^ΔΔCt method.

**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% CO2 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: CUCGUUGUGGUGGGACUCUAUG, si-Loc554202-2: GGAGCGCUUUGUGUGAGAAGUUGAA, si-Loc554202-3: GCAGGUAGAAGGUAUCCCGA. 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 1 × 10^4 cells/well. The following day, 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.

**Colonies 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 was 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
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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.

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

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

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
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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

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

FIGO, international federation of gynecology and obstetrics; HPV, human papilloma virus; HR, hazard ratio; CI, confidence interval.
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
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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 forming capability of the two cervical cancer cell lines, which indicated that Loc554202 is implicated 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, located on the mitochondrial outer 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 proapoptotic family member 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 downregulation 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.

References


Chakravarty, D., Shoner, A., Nair, S.S., Giannopoulou, E., Li, R.,


