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ORIGINAL ARTICLE

Aberrant expression profile of translationally controlled tumor protein and tumor-suppressive microRNAs in cervical cancer

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Summary

Purpose: *Invasive and recurrent cervical cancer accounts for major mortality among women. The activity of biomarkers in cervical cancer varies with different pathological stages. The purpose of the present study was to evaluate the expression of 2 biomarkers in cervical cancer and their possible contribution to novel therapeutic strategies.*

Methods: *In this study, we assessed the expression of translationally controlled tumor protein (TCTP) using immunohistochemistry and Western blot analysis. The expression pattern of miR-143 was also evaluated using Northern blot analysis.*

Results: *HeLa cells and mice were used for tumor induction. A group of mice injected with HeLa cells and incubated for 6 weeks developed initial tumor, while a different group of mice injected with HeLa cells and incubated for about 10 weeks developed advanced stage cervical cancer. Histological analysis revealed higher proliferation of cells resulting in complex forms of tumor in advanced cervical cancer, whereas cell clustering was not found to be initiated in the initial stage. The results of immunohistochemistry and Western blot analysis indicated less variation in the expression of TCTP, but significant difference was observed in advanced stage. Expression of Bax apoptotic protein was higher in the initial stage of the tumor than in the advanced cervical cancer. Similar pattern of marginal downregulation of miR-143 was observed between control and initial tumor stages, but striking reduction in miR-143 expression was observed in advanced stages of tumor development.*

Conclusion: *The results of this study reveal a new aspect of altered expression of biomarkers in different pathological stages that could help identify novel therapeutic strategies for cervical cancer treatment.*

Key words: *anti-Bax antibody, cervical cancer, HeLa cells, miR-143, translationally controlled tumor protein*

Introduction

Cervical cancer is one of the most prevalent forms of malignancy among women. The aggressive nature of cervical cancer is evidenced with the incidence of 500,000 new cases globally and 275,000 deaths per year [1]. In many developed countries, the occurrence of cervical cancer is lowered due to their established screening programs and health education [2]. However, upon recurrence after treatment or during metastatic stage, cervical cancer is very fatal [1]. Recently, the patient median overall survival has increased from 12 to 17 months by including bevacizumab in the standard chemotherapy [3] but the improvement in survival in advanced and recurrent disease remains less than 2 years. Hence, further studies are needed to provide more accurate understanding towards improving the existing strategies or evolving new strategies to treat this disease.

TCTP is a growth associated protein that is ubiquitously expressed in eukaryotes [4,5]. Al-

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though TCTP expression is found in most tissues, its expression differs extensively with different cell types [6] and developmental stages [7,8]. Recent studies have shown that TCTP plays an important role in the progression of cell cycle and tumor metastasis [9,10]. It is highly expressed in many cancer tissues, but its role in regulating tumor progression remains unclear [11]. Anti-apoptotic activity due to TCTP has also been reported [12]. Its role in the progression of cervical cancer and expression in different pathological conditions are yet to be clearly investigated.

MicroRNAs (miRs) are regulatory, small non-coding RNA that form base pairing with their complementary mRNA and suppress translation [13,14]. Recent studies on miRs have shown their role in apoptosis [15], differentiation [16] and cancer [17]. The role of miRs as tumor suppressors is well studied in different cancer types including liver [18], lung [19], breast [20], colon [21], pancreatic [22], gastric [23] and nasopharyngeal cancer [24]. The aberrant expression of different miRs in cervical cancer has been reported by Wang et al. [2]. Among them, miR-143 was found to be down-regulated in cervical cancer tissue [25].

In this study, the expression of TCTP and miR-143 was evaluated at various stages of cervical cancer using a cervical cancer induced mouse model.

Methods

Experimental animals

Nude athymic female mice (BALB/c-nu/nu) aged 6 weeks were chosen and subjected to various experimental analyses. All the experimental designs and studies were approved by the Institutional Ethics Committee that was specifically organized for this project. For experimental purposes, cervical cancer was induced in mice by injecting them HeLa cells. Initially, HeLa cells were grown in a serum-free medium and the obtained individual cells were resuspended in 100 µL of fetal bovine serum (FBS) and growth factor-free DMEM at a density of 1×10^6 cells. The mice were injected with 100 µL of HeLa cells at a density of $1x10^6$ subcutaneously into the left and right flanks. The mice were then housed in institutional animal care unit and monitored with care for the next 5-10 weeks. Mice that developed primary tumor were sacrificed on the 5th week post HeLa cells injection and those with advanced tumors were sacrificed on the 10th week postinjection.

Immunohistochemistry observations

Paraffin-embedded tissue sections were subjected to microtome sectioning (6 µm). The tissue sections were preheated and deparaffinized using xylene followed by rehydration with ethanol. The endogenous peroxidase activity was quenched by immersing the sections in freshly prepared 3% H_2O_2 in methanol for 20 min followed by heat-induced antigen retrieval in 10 mmol/L citrate buffer (pH 6.0). Nonspecific antigens were blocked by incubating the sections with 4% bovine serum albumin (BSA) for 1 h. The sections were then incubated with suitable primary antibody of anti-TCTP (ABCAM, ab37506, Cambridge, USA) or anti-Bax antibody (ABCAM, ab81083) followed by incubation with suitable secondary antibodies (anti-Rabbit IgG, Sigma Aldrich, A0545, Hong Kong). The targeted specific protein was then stained with DAB (diaminobenzidine) kit and the results were registered.

Western blot analysis

The protein samples from normal and cervical cancer tissues were resolved in 12% SDS-PAGE gel as previously described [26] and transferred to PVDF membrane. The membrane was then incubated with primary antibodies (anti-TCTP antibody, ABCAM, ab37506 or anti-Bax antibody, ABCAM, ab81083) following the manufacturer's instructions. After washing, the membrane was incubated with the respective secondary antibodies, and substrate was added to observe the hybridization signals.

Northern blott analysis

RNA was isolated as previously described [27], resolved in 12% gel, transferred onto nitrocellulose membrane and probed with a ^{32}P-labeled miR-specific oligo, as previously described [28]. The miR sequence information was collected from miRBase and suitable oligo probes were designed and the probe of antisense oligo for miR-143 was used. Membrane was stripped off and re-probed with antisense oligo of U6 snRNA which served as loading control.

Results

Mouse model with cervical cancer

The natural occurrence of cervical cancer in mice is rare to find and hence HeLa cells were inoculated to artificially induce cancer. After injection, the mice developed a primary tumor on the 5th week and advanced stage disease on the 10th week of inoculation. The dissected cervical tissue sections from control mice as well as from the tumor induced on the 5th and 10th week in mice were subjected to histological analysis and their results are shown in Figure 1A-C. Hematoxylin and eosin staining of the tissue sections helped differentiate the normal and cancer tissues. In the control tissue sections, nuclei were found to be more prom-

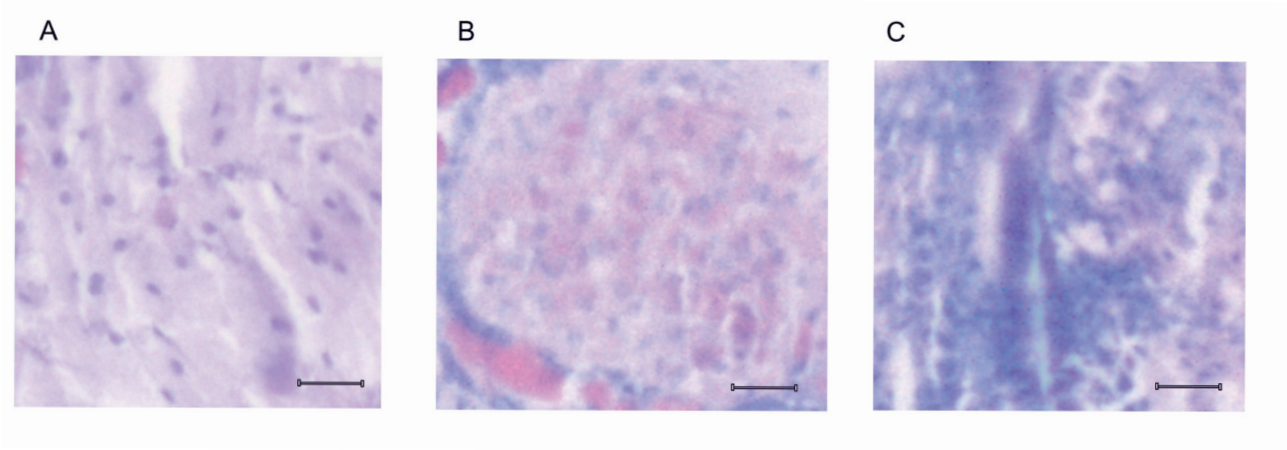

Figure 1. Histopathological analysis of cervical cancer. **A:** Normal cervical tissue with regular arrangement of cellular pattern. **B:** The mice injected with HeLa cells developed cervical tumor on the 5th week with higher proliferative cells. **C:** Histological section of advanced cervical tumor on the 10th week with clusters of more proliferative cells. Scale bar=20µm size.

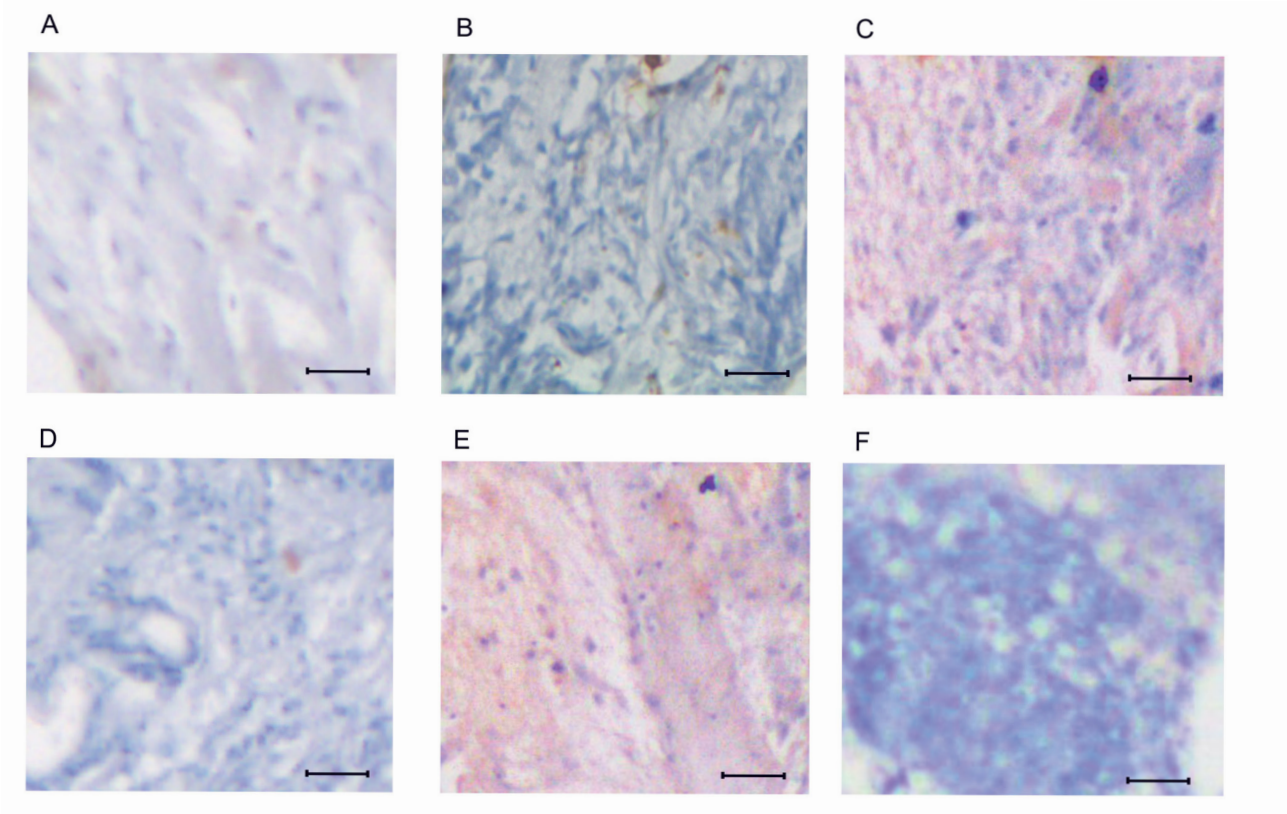

Figure 2. Immunohistochemical analysis of TCTP and apoptotic protein expression in cervical tumor. **A:** normal mouse cervical tissue stained with TCTP marker. **B:** Initial stage of cervical tumor (5th week) stained with TCTP marker. **C:** Immunohistochemical staining of advance tumor with TCTP marker. **D:** control mouse cervical tissue stained with anti-Bax antibody, an apoptotic marker. **E:** initial stage of cervical cancer with increased expression of apoptotic marker. **F:** Striking inhibition of apoptotic protein expression in advanced cervical cancer tissue. Scale bar = 50µm size.

inent and equally distributed (Figure 1A), while the tissue sections of the mice inocluated with HeLa cells for 5 weeks exhibited irregular tissue patterns with more replicating cells (Figure 1B). The tissue sections from the mice with advanced stage of cervical cancer displayed severe abnor-

malities with a heavy replication pattern of the nucleus along with clustered arrangement of cells (Figure 1C).

TCTP and apoptotic protein expression in cervical cancer

In order to evaluate the role of TCTP in developing cervical cancer, we analyzed the expression of TCTP in relation to the apoptotic response to assess its effective function. The normal and cancer tissues were subjected to immunohistochemical analysis using anti-TCTP and antiapoptotic antibodies (Figure 2A-F). The normal tissue showed less positive cells for TCTP (Figure 2A) and apoptotic signals (Figure 2D). During the initial stage of tumor development (5th week), there was not much variation in TCTP expression (Figure 2B) when compared with the control tissue (Figure 2A). More apoptotic signals were observed in the initial form of tumor progression (Figure 2E) when compared with their respective control (Figure 2D). However, during the advanced stage of cervical cancer, positive expression of TCTP was observed (Figure 2C). Interestingly, the apoptotic response in advanced stage of cancer development was suppressed as compared to early stage cancer (Figure 2F).

The results of immunohistochemistry analysis were further confirmed through Western blot analysis. TCTP expression in normal cells (Figure 3A, Lane 1) and in the initial stage of cervical cancer (Figure 3A, Lane 2) tissue showed less variation, but its expression obviously increased in the advanced stage of tumor development (Figure 3A, Lane 3). Similarly, the expression of anti-Bax protein as observed in the Western blot analysis correlated with the immunohistochemical observations (Figure 3B). The control tissue showed less expression of apoptotic proteins (Figure 3B, Lane 1) which was found increasing in the initial tumor (Figure 3B, Lane 2) and decreasing during the advanced stage of tumor development (Figure 3B, Lane 3). The β-actin antibody was used as a loading control in both the experiments.

Expression of miR-143 in normal and cancer tissue

Northern blotting was performed to understand the expression pattern of miR-143 in normal and cervical cancer tissue at different stages. The normal cervical tissue showed stable expression of miR-143 (Figure 4, Lane 1) but was found to be downregulated in the initial tumor stages (Figure 4, Lane 2). With the tumor progression,

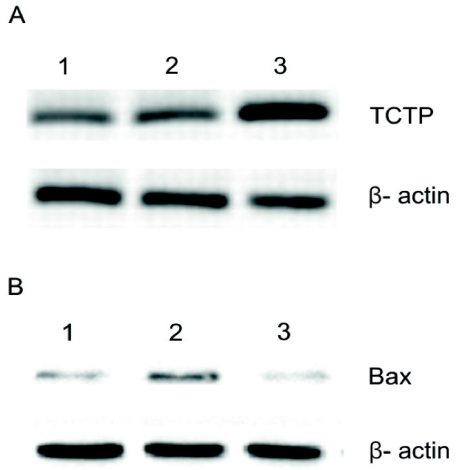

Figure 3. Western blot analysis of TCTP and anti-Bax expression in cervical cancer. **A:** Lane 1 shows the TCTP expression in normal tissue of cervical mouse model. Lane 2: initial tumor growth shows minimal expression pattern of TCTP over the control tissue. Lane 3 depicts the elevated expression pattern of TCTP protein in advanced stage of cervical tumor. **B:** Lane 1 shows the Western blotting of apoptotic protein in normal cervical tissue. Lane 2: Western blotting of apoptotic protein shows strong signals over the initial cervical tumor. Lane 3: Western blotting of apoptotic protein shows inhibited expression against high proliferative cervical tumor. For control, β-actin was used.

significant inhibition of miR-143 expression in the advanced stages was found (Figure 4, Lane 3).

Discussion

In this study, the expression of TCTP and miR-143 at different stages of cervical cancer was analyzed. In order to assess it, the mouse model system was used. Developing a suitable mouse model system helps mimic the different stages of cervical cancer with similar histopathological conditions that occurs in humans. Along with this, humans and mice exhibit identical expression pattern for many biomarkers [29,30]. The injected dose of HeLa cells was more successful to induce different stages of cervical cancer in optimum time range. Apart from HeLa cell line, other cell types like CaSki, ME-180, and SiHa cell lines can also be used [31].

The injected HeLa cells were able to form histologically distinguishable tissue pattern in the initial and advanced stages of cervical cancer (Figure 1A-C). A diverse cellular arrangement was observed during the 10th week of inocula-

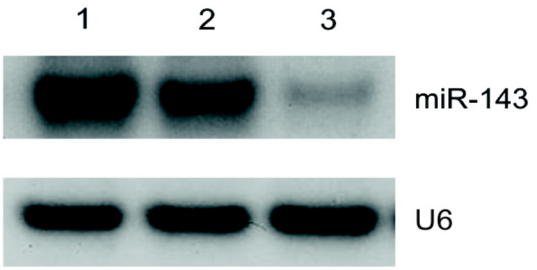

Figure 4. Northern blotting to determine miR-143 expression in normal tissue and cervical cancer. Lane 1 shows miR-143 expression level in the control cervical tissue. Lane 2 shows slightly inhibited expression level of miR-143 in initial cervical cancer. Lane 3 shows heavily inhibited pattern of miR-143 expression level in advanced cervical cancer tissue. U6 was used as a loading control.

tion with clusters of cancer cells (Figure 1C). The main goal of the present study was to find out the differential expression, if any, of TCTP and miR-143 during initial and advanced stages of cervical cancer. Aberrant expression of both TCTP (Figure 2A-C & Figure 3A) and miR-143 (Figure 4) at different stages of cervical cancer was noticed.

High expression of TCTP has been reported to suppress the expression of apoptotic proteins, leading thus to cancer development [32,33].

Expression of TCTP was observed to be altered significantly during advanced stages of cervical cancer (Figure 2C & Figure 3A) and not in the initial tumor stage. The results showed a correlation with the observations on apoptotic signals (Figure 2D-F & Figure 3B) which implies that TCTP expression suppresses the apoptotic signals in the advanced tumor stages. But in the initial tumor stage, more apoptotic signals favored the control of tumor progression without any significant upregulation of TCTP expression.

Conclusion

The results of this study suggest that the increased expression of TCTP favors rapid cell growth and it also increases the tumorigenic potential. The normal and initial tumor showed less variation in miR-143 expression, while it was completely suppressed during the advanced stage of cervical cancer.

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Authors' contributions

Dr. Li-juan Hou contributed in the concept, development and designing and executing the experiments. Dr. Jian-jun Zhai performed data interpretation and contributed in the experimental work.

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