Expression of HOXC10 gene regulates the growth and invasion of prostate cancer cells
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Abstract

The present study was undertaken to decipher the role of HOXC10 gene in regulating the growth and metastasis of prostate cancer. The results revealed significant (p<0.05) up-regulation of HOXC10 gene in human prostate cancer tissues and cell lines. The silencing of HOXC10 transcript level significantly (p<0.05) inhibited the growth and colony formation of DU145 and 22Rv1 prostate cancer cells. The DAPI staining revealed that inhibition of DU145 and 22Rv1 prostate cancer cell viability was due to the induction of apoptosis. The transwell assay showed that HOXC10 significantly (p<0.05) inhibits the invasion of prostate cancer cells. The Western blotting revealed that HOXC10 gene exerts its effects via modulation of Ras/Raf/MEK/ERK signaling cascade. Collectively, the results point towards the therapeutic potential of HOXC10 in the treatment of prostate cancer.

Introduction

Prostate cancer is one of the most prevalently detected solid cancers among the male population (Mehtälä et al., 2020). The incidence of prostate cancer is affected by family history, age, and human race (Pienta and Esper, 1993).

With recent advancements, there has been significant improvement in the treatment of prostate cancer. However, the clinical outcome is still far from the descent. Additionally, frequent relapse and distant metastasis makes it even more difficult to manage (Di Vizio et al., 2012). Hence, to develop more effective treatment regimens the molecular mechanisms governing the development of prostate cancer need to be thoroughly understood.

The homeobox (HOX) genes function in normal human embryonic development and are crucial for eukaryotic cell differentiation (Van Oostveen et al., 1999). The HOX gene products function as transcription factors and are characterized by the presence of a homeodomain region which assists them in attaching to the promoters of the target genes to enforce the transcription of target genes (Pearson et al., 2005). The homeodomain is 61 amino acid long stretch of HOX transcription factors with an extremely high level of sequence conservation (Jolma et al., 2013). The genetic mutations of HOX genes in humans have been reported to act among the vital cues for the onset of human cancers (Bhatlekar et al., 2014). The HOXC10 gene has been reported to be involved in the growth of some human cancers (Pathiraja et al., 2014; Zhai et al., 2007, Ansari et al., 2012). However, limited information is available regarding the function of HOXC10 in controlling the molecular mechanics of human prostate cancer.

As such, the present study was designed to investigate the regulatory function of HOXC10 proliferation and metastasis. Herein, we report that HOXC10 is significantly up-regulated in prostate cancer and regulates its proliferation and development via modulation of Ras/Raf/MEK/ERK signaling pathway.
Materials and Methods

Clinical specimens and cell line maintenance

A total of 25 pairs of prostate specimens and the normal adjacent tissues were obtained under the standard ethical guidelines from the prostate cancer patients admitted into the Hospital. The tissues were fixed using formaldehyde and transported in a liquid nitrogen container. The tissues were stored at ultra-low temperatures till further experimentation.

Cell lines and culture conditions

The normal human prostate epithelial cell line (hTERT EP156T) and the prostate cancer cell lines (DU145, NCI-H660, 22Rv1 and AT3B1) were purchased from the American Type Collection Center (USA). All the cell lines were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) medium (Thermo Scientific) supplemented with 4.5% D-glucose and L-glutamine plus 10% fetal bovine serum (FBS). The growth medium also contained 1% penicillin/streptomycin and 0.8% gentamicin. The cell lines were maintained in a humidified incubator at 37°C with 5% CO2 saturation. The RNA was isolated from the prostate tissues and cell lines with the TRIzol reagent (Thermo Scientific). The RNA was then treated with DNase I and used for cDNA synthesis. The cDNA synthesis was performed by first strand cDNA synthesis kit protocol (Takara) by the help of TRIzol reagent (Thermo Scientific). The cDNA synthesis was performed by first strand cDNA synthesis kit protocol (Takara) by following the manufacturer guidelines. The SYBR Green mix (Thermo Scientific) was used to per-form the quantitative real-time PCR (qRT-PCR) using the gene-specific primers of HOXC10 were

Forward 5′-TGACTCCAATTGCAGGTGA-3′ and Reverse 5′-ACTAGGGTTGAGCGACAGG-3′.

The estimation of expression levels of HOXC10 was made with the help of 2-ddct method with actin as a control.

Proliferation assay

The proliferation of the DU145 and 22Rv1 cells was determined by MTS assay as described previously (Arab-Bafari et al., 2016). Breifly, the transfected prostate cancer cell lines, DU145 and 22Rv1 were added at the density of 10^4 in 150 µL of growth medium and cultured for different time durations at 37°C in the wells of 96-well plate. Afterward, 20 µL of 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxy-xyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium solution (MTS, Promega) were added to each well at indicated time points of cell culture (0, 20, 40, 60, 80 and 100 hours post-inoculation) following which the absorbance was measured at 470 nm wavelength after 2.5 hours incubation at 37°C.

Clonogenic assay

The effects of HOXC10 silencing on the colony formation of DU145 and 22Rv1 were evaluated by clonogenic assay as described previously (Yu et al., 2020). The transfected cancer cells were grown in the 6-well plates for 2 weeks at 37°C. The cells were subsequently collected, fixed with ethanol and then stained with 0.1% crystal violet (Merck) stain. The light microscope was then used for visualizing the cancer cell colonies and the colony number was presented as a percent of control.

4’,6-Diamidino-2-phenylindole (DAPI) staining assay

DAPI staining was performed to detect the apoptotic cells as described previously (Yu et al., 2017). Briefly,

Box 1: Cell Invasion Assay

Principle

The Boyden chambers were used to determine the invasion of the transfected prostate cancer cells.

Requirements

Boyden chamber (8 µm pore size fitted with Matrigel; Costar, USA); Bright field microscope; Crystal violet (0.1%; Sigma-Aldrich, USA); Dulbecco’s Modified Eagle’s medium Gibco, USA; Paraformaldehyde (4%; Sigma-Aldrich, USA); Prostate cancer cells [(DU145; 22Rv1); ATCC, USA]]

Procedure

Step 1: The Dulbecco’s Modified Eagle’s medium (500 µL) containing 10^5 Prostate cancer cells was added to the upper portion while the lower portion/chamber was supplemented with only the Dulbecco’s Modified Eagle’s medium.

Step 2: The culturing of cells was performed for 48 hours at 37°C in the Boyden chambers.

Step 3: The cells invading the lower portion of the chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and then visualized with the help of bright field microscopy.

Step 4: At least 10 random fields were used for counting the invading cells and relative invasion of the cancer cells was presented as a percent of control.

Reference

Jin et al., 2018

Reference (video)

Zheng et al., 2017
the transfected prostate cancer cells were cultured at 37°C in 6-well plates for 24 hours. The cells were then harvested and washed with phosphate-buffered saline (PBS). Methanol was used to fix the cells and fixed cells were subsequently stained with DAPI solution (Thermo Scientific). Afterward, the nuclear morphology of the cells was examined under the fluorescence microscope.

**Immunohistochemical analysis**

The expression study of HOXC10 was assessed by immunohistochemical analysis of clinical tissues, as described previously (Chen et al., 2016). Briefly, Tumor tissue samples from xenograft tumor assay were embedded in paraffin and cut after fixing in 4% paraformaldehyde. Then consecutive sections at 4 µm thick were used for immunohistochemical assay using anti-HOXC10 (Santa Cruz Biotechnology, Inc., USA). The expressions were grouped into negative, positive but weak and strong positive for no staining, light brown staining and dark brown staining cells, respectively. The evaluation of the histochemical staining was done by three pathologists blind to the study.

**Western blotting**

Western blotting was performed to examine the protein expression as described previously (Yu et al., 2017). Briefly, total proteins were isolated from the prostate cancer cell lines, DU145 and 22Rv1, transfected with si-HOXC10 or si-NC after 72 hours of transfection with the help of RIPA lysis buffer (Thermo Scientific). The quantification of extracted proteins was estimated with Pierce™ BCA protein assay kit (Thermo Scientific). Around 45 µg of extracted proteins were denatured at 95°C for 10 min in Lammli buffer (Biorad) containing β-mercaptoethanol (Sigma). The denatured proteins were then run on 10% SDS-PAGE. Using 5% skimmed milk, the PAGE gel was blocked and contents blotted to PDF membrane which was then exposed to specific primary antibodies following which secondary antibody exposure was given to the membrane. Finally, the protein bands were visualized, and their concentration was determined with the chemiluminescence method. The Western blotting of proteins of interest was normalized with the help of human actin protein.

**Statistical analysis**

The analysis of statistical data was made with the help of GraphPad Prism 7.0 software by performing one-way ANOVA and student’s t-test. The values were presented as mean ± standard deviation (SD) determined from the three experimental replicas. A p value of <0.05 was taken as the measure of the statistically significant difference.

**Results**

**HOXC10 expression in prostate cancer**

The expression of HOXC10 was analyzed in different prostate tissue specimens and prostate cancer cell lines. It was observed that prostate cancer tissues exhibited significantly (p<0.01) higher HOXC10 expression (Figure 1A). The IHC analysis also revealed HOXC10 protein expression to be considerably higher in prostate cancer tissues than normal tissues (Figure 1B). Similarly, prostate cancer cell lines (DU145, NCI-H660, 22Rv1 and AT3B1) were found to possess significantly (p<0.05) higher transcript levels of HOXC10 in comparison to the normal epithelial prostate cell line (hTERT EP156T) (Figure 1C). Together, the results are supportive that HOXC10 gene expression is enhanced in prostate cancer and thus might be involved in regulating the mechanics of this deadly disorder. Among the cancerous cell lines, the DU145 and 22Rv1 cell lines showed comparatively higher HOXC10 expression and were thus taken for further study.

**HOXC10 silencing in prostate cancer cell proliferation**

The silencing of HOXC10 in DU145 and 22Rv1 was attained by transfecting the cancer cells with HOXC10 RNAi construct (si-HOXC10) and was confirmed by qRT-PCR and Western blotting both of which showed a significant (p<0.05) decrease of HOXC10 expression (Figure 2AB). When the proliferation of si-HOXC10 cancer cells was evaluated against those of the control cells, it was found that si-HOXC10 transfected cells exhibited significantly (p<0.05) lower proliferation rates (Figure 2C). The anti-cancer effects of HOXC10 gene silencing were also evident from the decrease in the cell proliferation via induction of apoptosis (Figure 4). Taken together, the results suggest that repression of HOXC10 in prostate cancer cells effectively declined the cell proliferation via induction of apoptosis.

**HOXC10 silencing in invasion of cancer cells**

The invasion of DU145 and 22Rv1 prostate cancer cells transfected with si-NC or si-HOXC10 was estimated by transwell assay. It was found that silencing of HOXC10 gene exhibited significantly (p<0.05) lower invasion rates in comparison to the control transfected cells (Figure 5). The invasion of cancer cells was only 29 and 25%, respectively, for DU145 and 22Rv1 cells transfected with si-HOXC10. The results indicate that HOXC10 is active in regulating the metastasis of prostate cancer and thus might be employed as an important molecular target for restricting the growth and proliferation of prostate cancer.

**Ras/Raf/MEK/ERK in prostate cancer**

The results showed that silencing of HOXC10 gene reduced the levels of Ras and Raf proteins and blocked the phosphorylation of MEK and ERK proteins (Figure...
Figure 1: HOXC10 gene expression is upregulated in prostate cancer. (A) qRT-PCR expression analysis of HOXC10 gene in normal and cancerous tissue specimens; (B) IHC analysis showing expression of HOXC10 in prostate cancer and normal tissues; (C) expression of HOXC10 in normal epithelial prostate cell line (hTERT EP156T) and the cancer cell lines (DU145, NCI-H660, 22Rv1 and AT3B1). The experiments were performed in triplicates and expressed as mean ± SD (*p< 0.05)

Figure 2: Transcriptional silencing of HOXC10 reduced the growth of prostate cancer cells effectively. (A) qRT-PCR expression of HOXC10 gene in DU145 and 22Rv1 prostate cancer cell lines transfected with si-NC or si-HOXC10; (B) Western blotting showing protein expression of HOXC10 gene in DU145 and 22Rv1 prostate cancer cell lines transfected with si-NC or si-HOXC10; (C) MTS assay showing proliferation of DU145 and 22Rv1 prostate cancer cell lines transfected with si-NC or si-HOXC10. The experiments were performed in triplicates and expressed as mean ± SD (*p<0.05)
However, the expression of non-phosphorylated MEK and ERK proteins were found to be unaffected under HOXC10 gene silencing.

**Discussion**

The HOX genes regulate the differentiation and identity determination of different eukaryotic cells and tissues (Akam, 1995). Following their first identification from the common fruit fly (*Drosophila melanogaster*), the HOX genes were identified from many species of animals including mammals (Favier and Dolle, 1997). The HOX genes code for homeodomain transcription factors which are primarily expressed in embryonic tissues (Martinez and Amemiya, 2002).
Different human cancer tissues are regulated by the HOX genes (Shah and Sukumar, 2010). The human HOX genes are recognized in four different clusters (A, B, C and D) located on chromosomes 7, 17, 12 and 2, respectively (Scott, 1992). The HOX genes are further divided into 13 paralogous groups based on their sequence similarity (Mallo et al., 2010). The HOXA10, HOXC10 and HOXD10 fall in the same paralogous group (Hostikka et al., 2009). Human HOXC10 gene expression level was found to be elevated in many human cancers as deduced by previous studies (Zhai et al., 2007; Feng et al., 2015). Our results also support the similar correlation (Zhai et al., 2007; Feng et al., 2015). The HOXC10 overexpression was observed in both the prostate cancer tissues and the cell lines. As stated in the beginning, the HOX genes regulate the mechanics of different cancers confirming our findings that the proliferation of human prostate cancer cells was affected by HOXC10 gene.

The down-regulation of HOXC10 negatively affected the proliferation of prostate cancer cells suggesting its direct involvement in prostate cancer growth. Interestingly, the HOXC10 gene was found to affect the colony formation of prostate cancer cells. Similar results were obtained from the previous research studies (Guo et al., 2017). The human HOXC10 gene was deduced to regulate the invasiveness of the human cervical cancer cells as reported elsewhere (Lopez et al., 2006). In agreement with this study, it was revealed that the repression of HOX10 gene reduced the invasion of prostate cancer cells.

The cancer cells proliferate at very enhanced rates, the basis of which lies at the elevated expression of a number of signaling pathways (Schulze and Harris, 2012). These pathways include the Ras/Raf/MEK/ERK signaling pathway.

Figure 6: Silencing of HOXC10 gene inhibited the Ras/Raf/MEK/ERK signaling pathway. (A) Western blotting of Ras, Raf, MEK, p-MEK, ERK and p-ERK proteins from DU145 prostate cancer cell lines transfected with si-NC or si-HOXC10. (B) Western blotting of Ras, Raf, MEK, p-MEK, ERK and p-ERK proteins of 22Rv1 prostate cancer cell lines transfected with si-NC or si-HOXC10. The experiments were performed in triplicates and expressed as mean ± SD (*p<0.05)
signaling cascade (Chen et al., 2016; Montagut and Settleman, 2009). The Ras/Raf/MEK/ERK pathway has been shown to be aberrantly activated in several human cancers such as ovarian cancer (Ma et al., 2018) and melanoma (Riveros et al., 2017).

**Conclusion**

The down-regulation of HOXCI0 obstructs the Ras/Raf/MEK/ERK signaling pathways in prostate cancer cells to exert the growth inhibitory effects evident as decline of cancer cell proliferation and colony formation.

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**Ethical Issue**

The study was approved by the Research Ethics Committee of Shanghai Sixth People's Hospital East affiliated to Shanghai University of Medicine and Health Science, Shanghai, China under approval No. SUM-232HT-2019. Informed written consent was obtained form the patients.

**Conflict of Interest**

Authors declare no conflict of interest

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