Cholinergic α5 nicotinic receptor is involved in the proliferation and invasion of human prostate cancer cells

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Abstract. Nicotinic acetylcholine receptor (nAChR) subunit α5 (α5-nAChR) is involved in tumor cell proliferation, inhibition of apoptosis, progression of metastasis, and induction of angiogenesis in certain solid tumors. However, the role of α5-nAChR in prostate cancer cell growth and metastasis is unclear. In the present study, the role of α5-nAChR in cell proliferation, migration, invasion and apoptosis was investigated by silencing the expression levels of α5-nAChR in the prostate cancer cell lines DU145 and PC3. A siRNA oligonucleotide targeting α5-nAChR was designed. The cell proliferation of DU145 and PC3 cell lines was analyzed by the Cell Counting Kit-8 (CCK-8) assay. Cell migratory and invasive activities were determined using wound healing and Transwell assays, respectively. Western blot analysis was used to quantify α5-nAChR, p-AKT and p-ERK1/2 levels in DU145 and PC3 cells. Knockdown of α5-nAChR was associated with decreased cell proliferation, migration, invasion and increased apoptosis. In addition, decreased phosphorylation levels of AKT and ERK1/2 were revealed following α5-nAChR knockdown in DU145 and PC3 cells compared with those observed in the scramble control samples. The expression levels of the apoptosis-related proteins were altered following silencing of α5-nAChR. In summary, the data indicated that α5-nAChR was involved in the proliferation and invasion of human prostate cancer cells.

Introduction

Prostate cancer is one of the most common malignant tumors and is ranked as the second cause of cancer-associated deaths in male patients in developed countries (1). Prostate cancer mainly develops due to a switch from androgen-dependent to androgen-independent growth (2). Nicotinic acetylcholine receptors (nAChRs) comprise ligand-gated ion channels, which consist of 5 subunits with 4 transmembrane domains in each subunit. The nicotinic acetylcholine receptor (nAChR) signaling pathway affects various cancer-associated pathways that are important in promoting tumor cell proliferation, metastasis and angiogenesis (3-8).

It has been revealed that the α5 subunit of the nAChR (α5-nAChR), a member of the nAChR subunit family, is closely associated with the incidence of lung cancer. The activation of α5-nAChR is involved in tumor growth and metastasis and is important in the formation, metastasis and recurrence of lung cancer (9,10). Previous studies have demonstrated that α5-nAChR is associated with gastric cancer (11-17). For example, exposure of nicotine to gastric cancer cells inhibits the cisplatin-induced apoptosis via the α5-nAChR/AKT signaling pathway (11). However, it is unknown whether α5-nAchR is involved in the progression of prostate cancer. Based on the aforementioned findings, the involvement of α5-nAChR in the incidence and progression of prostate cancer was examined. Therefore, in the present study, the expression levels of α5-nAChR were examined in prostate cancer and normal prostate tissues. The expression levels of α5-nAChR were upregulated in prostate cancer tissues. Furthermore, silencing of α5-nAChR expression significantly inhibited prostate cancer cell invasion and migration in vitro. In addition, previous studies have identified the downstream signaling pathways of α5-nAChR, which are involved in prostate cancer progression.

Materials and methods

Cell culture. The human normal prostate epithelial cell line RWPE-1 and the androgen-independent prostate cancer cell lines PC3 and DU145 were used in the present study. The metastatic potential of DU145 is higher than that of the PC3 cell line. 293 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. DU145 cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with a combination of 1% penicillin and streptomycin in the presence of 10% fetal bovine serum (Gibco). Ham's F-12K (Kaighn's) Medium (Gibco; Thermo Fisher Scientific, Inc.) was used to culture
PC3 cells with 1% penicillin and streptomycin and 10% fetal bovine serum in the culture medium. 293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) with a combination of 1% penicillin and streptomycin and 10% fetal bovine serum. All the cells were incubated in standard cell culture conditions with 5% CO₂ and 95% humidity at 37°C.

Cell transfection. A siRNA oligonucleotide targeting α5-nAChR mRNA was synthesized by Shanghai GenePharma Co. Ltd. The sequence was as follows: 5'-CCC GCA AAC UAC GCT CCC TGT TGC ACT CA. A pair of scrambled control siRNAs with sequences different from those of the siRNA-α5-nAChR was designed. The pair of sequences was not homologous to any sequences found in GeneBank. When the cells reached 70-80% confluence, the transfection was conducted according to the transfection instructions. The cells were subsequently cultured under normal conditions for 36 h at 37°C.

Tissue samples from patients and immunohistochemistry (IHC). IHC was performed in 8 normal prostate tissues (all male, aged 66.37±4.53) and the results were compared with the analysis performed in 36 prostate cancer samples (Table I). The clinicopathological variables of these patients were collected. Written informed consent forms were obtained from the subjects. The study protocol was approved by the Research Ethics Committee of the Second Hospital of Hebei Medical University. IHC was performed to determine α5-nAChR expression. Paraffin-embedded tissue sections (5-μm thick) were deparaffinized with xylene, followed by rehydration using a graded series of 100, 90, 80 and 70% ethanol. Then, intrinsic peroxidase was deactivated with 0.3% H₂O₂ and intrinsic biotin was deactivated with skim milk. The sections were reacted with the primary (cat. no. ab166718; Abcam) and secondary (cat. no. ab97048; Abcam) antibodies. Finally, H₂O₂ was added to DAB to undergo reaction. Sections were then stained with methyl green, and the target proteins were observed under a light microscope (magnification, x100 and x400).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (CW BIO) was used to isolate total RNA as determined by the manufacturer’s instructions. A total of 500 ng of RNA was extracted from each sample and reverse-transcribed using the Reverse Transcription Reaction Kit (CW BIO). The relative mRNA levels of α5-nAChR, and β-actin (control) were determined by RT-qPCR using an FTC-3000 Real-Time PCR System. All real-time PCR assays used the SYBR Green Supermix. The cycles used for RNA amplification included a pre-denaturing step at 95°C for a duration of 10 sec, followed by 40 PCR cycles consisting of 5 sec at 95°C, 30 sec at 60°C, and 10 min at 72°C. All samples were repeatedly assayed in triplicate in each experiment. The relative amount of mRNA was determined by the comparative ΔCq method (18) and then normalized to the β-actin mRNA levels. The sequences of α5-nAChR primers were as follows: Forward, GA CTTCA CCCGGCA AACTACA and reverse, TTT GCTTCCC GTTGGCACTCA.

Western blotting. The cells were treated with PBS and subsequently lysed in RIPA buffer (Beyotime Institute of Biotechnology). Then the protein concentration was determined by the Pierce BCA Protein Assay (Thermo Fisher Scientific, Inc.). In each sample, 44 μg protein was resolved by gel electrophoresis using 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The proteins were transferred to a PVDF membrane, and finally analyzed by western blotting. The primary antibodies against α-tubulin (cat. no. ab18251) and anti-α5-nAChR (cat. no. ab166718) (rabbit polyclonal antibody) and the secondary alkaline phosphatase-coupled anti-rabbit IgG antibody (cat. no. ab97048) were obtained from Abcam. The membranes were blocked in 5% fat-free milk in TBS containing 0.1% Tween-20 at room temperature for 1 h and subsequently incubated with primary antibodies against tubulin (1:10,000 dilution) and α5-nAChR (1:1,000 dilution) for 2 h. The membranes were further incubated with secondary antibodies (1:5,000) for 1 h. The western blot assay was repeated 3 times in order to evaluate the repeatability of the procedure. Finally, the labeled proteins were detected by chemiluminescence (ECLPlus; Amersham Pharmacia Biotech; GE Healthcare) and analyzed using ImageJ software (v1.43; National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay. PC3 and DU145 cells were cultured in a 96-well plate at a density of 1,000 cells/well. The cells were divided into the control (si-RNA-NC, scramble sequence) and the test groups (si-α5-nAChR). The cell proliferation assay was performed as follows: A total of 10 μl CCK-8 solution (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well and the plate was incubated at 37°C for 2 h. A microplate reader was used to measure the absorbance at 450 nm.

Transwell assay. The cell migration assay was conducted using Transwell chambers. The Transwell inserts were placed in 24 well plates (Corning, Inc.). Following treatment with either full-length α5-nAChR plasmid or α5-nAChR-specific siRNA, 5x10⁵ cells/well were placed in the upper chamber with 200 μl culture medium (RPMI-1640 medium for DU145 and Ham's F-12K for PC3). The lower chamber was filled with 600 μl of 10% FBS culture medium (RPMI-1640 medium for DU145 and Ham's F-12K for PC3). The cells were then cultured for 24 h. The outer surface was subsequently washed three times with PBS, fixed with methanol for 20 min and stained with 0.2% crystal violet for 15 min at room temperature. The images were obtained immediately after drying. The number of migrated cells were counted in five randomly microscopic fields of view (Olympus Corp.) at x400 magnification. The experiments were repeated three times and the data were summarized.

TUNEL assay. One-Step TUNEL kit (Beyotime Institute of Biotechnology) was used following the manufacturer’s recommendations. Briefly, PC3 and DU145 cells were exposed to si-RNA-NC (scramble sequence) or si-α5-nAChR for 24 h and then fixed in 4% paraformaldehyde for 10 min at room temperature. Subsequently, the cells were washed with PBS three times and permeabilized for 2 min on ice and then the cells were resuspended in TUNEL working solution. Following incubation for 1 h in a humidified atmosphere at 37°C in the dark, the cells were counterstained with DAPI.
Table I. Expression of α5-nAChR in prostate tissue from prostate cancer patients.

| Clinical characteristics | Case no. (n) | α5-nAChR low expression (n) | α5-nAChR high expression (n) | P-value |
|--------------------------|-------------|-----------------------------|-----------------------------|---------|
| Age                      |             |                             |                             |         |
| ≤60                      | 15          | 8                           | 7                           | 0.5348  |
| >60                      | 21          | 9                           | 12                          |         |
| Tumor diameter (cm)      |             |                             |                             |         |
| ≤3.0                     | 19          | 7                           | 4                           | 0.1907  |
| >3.0                     | 17          | 10                          | 15                          |         |
| Lymph-node metastasis    |             |                             |                             |         |
| Yes                      | 4           | 2                           | 2                           | 0.906   |
| No                       | 32          | 15                          | 17                          |         |

α5-nAChR, nicotinic acetylcholine receptor subunit α5.

staining solution for 5 min at room temperature, DAPI was used for the nuclear staining and afterwards washed with PBS. The staining solution consisted of a mixture of methanol and DAPI as follows: 1 ml methanol and 2 µl DAPI (from a stock solution of 1 mg/ml). The mounting medium was S2100 (Solarbio Science and Technology Co., Ltd.). The number of TUNEL-positive cells were counted in five randomly fluorescence microscopic fields of view. The experiments were repeated three times and the data were summarized.

Wound healing assay. The cells were transfected by siRNA targeting α5-nAChR mRNA using NanoFectin Transfection Reagent. Following 24 h of incubation, a scratch was made to the bottom of the culture dish by the tip of a glass micropipette in order to establish a clean wound area. The cultured cells were maintained in their original culture medium. The culture dish with the scratched wound was photographed at the following time-points: 0, 12 and 24 h after the wound was made.

Statistical analysis. The data were expressed as the mean ± SD and were analyzed using SPSS v17 (SPSS, Inc.). Pearson’s chi-squared test was performed to examine the correlation of α5-nAChR expression with the various clinical factors. One-way ANOVA was performed to determine the differences between the experimental groups, and the least significant difference (LSD) post hoc test was used. A P-value <0.05 (P<0.05) was considered to indicate a statistically significant difference.

Results

α5-nAChR is overexpressed in prostate cancer. To investigate the expression levels of α5-nAChR in prostate cancer tissues, RT-PCR was initially used and the α5-nAChR mRNA levels were analyzed in 2 human prostate cancer cell lines. The results were compared with the human prostate epithelial cell line RWPE-1. The mRNA levels of α5-nAChR in the prostate cancer cell lines (PC3 and DU145) were higher than those noted in the normal prostate epithelial cell line (RWPE-1) (Fig. 1A). α5-nAChR protein expression was further assessed in the PC3 and DU145 cancer cell lines and in the normal prostate epithelial cell line RWPE-1 by western blotting. A similar expression pattern was noted to that observed for the mRNA expression (Fig. 1B and C).

Subsequently, IHC analysis was performed to examine the α5-nAChR expression levels in a tissue array consisting of 8 normal prostate tissues compared with those noted in 36 prostate cancer samples (Fig. 1D). The expression levels of α5-nAChR in prostate cancer tissues were increased (P=0.0384). Subsequently, the correlation between the levels of α5-nAChR expression and the clinicopathological variables of prostate cancer patients was evaluated. The expression levels of α5-nAChR were not associated with the parameters age, lymph-node metastasis and tumor diameter (Table I).

α5-nAChR promotes proliferation and migration of human prostate cancer cells. The initial experiments demonstrated that the α5-nAChR expression levels were different between prostate cancer and normal tissues. Additional experiments focused on identifying whether α5-nAChR could promote the proliferation and migration of prostate cancer cells and therefore contribute to the progression of prostate cancer. To determine the role of α5-nAChR in the biological behavior of prostate cancer cells, siRNA sequences of α5-nAChR were transfected into 3 cell lines (PC3, DU145 and RWPE-1). RT-qPCR was used to detect the transfection efficiency. si-α5-nAChR caused a significant inhibition in the expression levels of α5-nAChR in PC3, DU145 and RWPE-1 cells (Fig. 2A).

The effect of α5-nAChR on the growth of prostate cancer cells was determined using a CCK-8 assay. The proliferation of DU145 cells transfected with α5-nAChR-specific siRNA was significantly reduced (Fig. 2B). The effects of α5-nAChR-siRNA on DU145 cell proliferation indicated successful transfection of α5-nAChR siRNA in prostate cancer cells in vitro. Clone formation assays of PC3 and DU145 cells indicated that the clone number of the si-α5-nAChR-treated cells decreased significantly compared with that of the NC cells (Fig. 2C and D).

Subsequently, the wound healing assay indicated that overexpression of α5-nAChR markedly promoted cell migration into the wound areas compared with that of the negative control (NC) cells. Downregulation of α5-nAChR by si-RNA significantly inhibited the migratory activities of DU145 cells that expressed high levels of α5-nAChR (Fig. 3A and B).
The migratory activity of the cells transfected with either full-length α5-nAChR plasmid or α5-nAChR-specific siRNA was evaluated. When the cells were transfected with the α5-nAChR vector, their migratory activity into the lower chamber was increased (Fig. 3C and D). The cells transfected with α5-nAChR-specific siRNA demonstrated reduced migratory activity into the lower chamber. This finding suggested that α5-nAChR could promote the migratory activity of the DU145 prostate cancer cells.

α5-nAChR inhibits the induction of apoptosis of human DU145 and PC3 cells. The induction of apoptosis was detected by the TUNEL assay. The experimental results indicated that downregulation of α5-nAChR with siRNA-α5-nAChR increased the number of apoptotic cells compared with that of the control group (P<0.05, Fig. 4A and B).

Since cleaved caspase-3 expression is positively associated with cell apoptosis, the role of α5-nAChR in cell apoptosis was detected by western blot analysis of caspase-3 and cleaved caspase-3. The expression levels of total caspase-3 and cleaved caspase-3 were determined in DU145 and PC3 cells treated with control si-RNA-NC and siRNA-α5-nAChR. Silencing of α5-nAChR expression by siRNA-α5-nAChR decreased the expression levels of total caspase-3 and
increased the expression levels of cleaved caspase-3 (P<0.05) (Fig. 4C and D).

Downregulation of α5-nAChR expression alters the phosphorylation status of ERK1/2 and AKT. It has been revealed that upon binding to nicotine, nAChRs activate the ERK and PI3K/AKT signaling pathways in several human cancer cells. The levels of total ERK1/2 and phosphorylated ERK1/2 and levels of total AKT and phosphorylated were analyzed following transfection of DU145 and PC3 cancer cells with siRNA-α5-nAChR (Fig. 5A and B). The phosphorylation levels of p-ERK1/2 and p-AKT were significantly decreased by downregulation of α5-nAChR.
α5-nAChR (Fig. 5C and D). The results indicated that phosphorylation of ERK and AKT proteins was involved in the α5-nAChR-mediated abnormal growth of prostate cancer cells.

Discussion

Smoking is an independent risk factor for several tumors and has further been studied as a risk factor for prostate cancer (19-22). However, the results are inconsistent. Some studies have revealed that the risk of prostate cancer increases with increasing smoking frequency, which is considered an independent risk factor for this disease (23-25). In contrast to these findings, a number of studies have revealed no significant correlation between the smoking incidence and the incidence of prostate cancer, although several studies have revealed a high mortality percentage due to prostate cancer in patients who smoke (26-28). A possible explanation may be the presence of specific compounds in cigarettes that do not necessarily lead to
the cancer, but affect the cellular behavior of cancer cells and accelerate the progression of cancer. According to previous studies, it is speculated that nicotine, which is a carcinogenic compound found in cigarettes, may cause cancer progression by mimicking the biological function of acetylcholine and by binding to the nAChR, which subsequently causes a series of signaling cascades within the cell. α5-nAChR is a regulatory subunit of nAChR and is involved in the growth and metastasis of solid tumors, such as lung and gastric cancers. This subunit is a promising therapeutic target for human tumors. In the present study, it was speculated that α5-nAChR may be associated with the progression of prostate cancer.

Initial experiments demonstrated that α5-nAChR expression was increased in prostate cancer cell lines and cancer tissues compared with that observed in normal prostate cell lines and the corresponding adjacent tissues. The molecular mechanism underlying the upregulation of α5-nAChR in prostate cancer is complex. The gene cluster encoding nAChR and CHRNA5/A3/B4 is located on the 15q24-25 region and is co-expressed in a variety of cell types (29,30). The promoter region contains a GC structure, which has multiple transcription factor binding sites. The transcription of these 3 genes may be regulated by various transcription factors (31). Notably, the transcriptional direction for α5-nAChR is opposite to that of the A3 and B4 subunits, indicating that in addition to regulating the entire gene cluster, a distinct transcriptional regulatory mechanism may exist for α5-nAChR expression. The mechanism of the regulation of α5-nAChR expression is poorly understood compared with the evidence presented on the other subunits of nAChR.

In the present study, the data confirmed that α5-nAChR could promote the proliferation and migration of prostate cancer cells. The opposite effect was noted for the induction of apoptosis, which was inhibited in prostate cancer cells. The function of α5-nAChR in tumors is not widely studied compared with the other subunits of nAChR. To the best of our knowledge, α5-nAChR is upregulated and can promote tumor growth and metastasis in lung and gastric cancers. The

Figure 4. Knockdown of α5-nAChR induces apoptosis of human prostate cancer cells. (A) A TUNEL assay was performed to detect the number of apoptotic cells in the NC and si-α5-nAChR groups. Representative fluorescence micrographs are presented. (B) The percentage of apoptotic cells was increased when the expression levels of α5-nAChR were suppressed. (C) The expression levels of the apoptosis-related proteins were confirmed using western blotting. (D) Statistical analysis of western blotting results was performed using the ImageJ software. *P<0.05. α5-nAChR, nicotinic acetylcholine receptor subunit α5; NC, negative control.
results from previous studies are consistent with our findings. In the present study, silencing of α5-nAChR inhibited only the proliferation of the prostate cancer cell line DU145 and exhibited little or no effect on the proliferation of the prostate cancer cell line PC3. Both PC3 and DU145 cell lines are androgen-independent prostate cancer cell lines. However, their cellular response to exogenous stimuli may be distinct. For example, the metastatic potential of DU145 cells is higher than that of PC3 cells. Knockdown of α5-nAChR did not influence the proliferation of PC3 cells but inhibited cell proliferation of DU145 cells. The data indicated that α5-nAChR played a significant role in promoting cell proliferation of certain prostate cancer cell types.

The data demonstrated that α5-nAChR promoted the development of prostate cancer by activating the PI3K/AKT signaling pathway, which has been revealed to play an important role in cancer progression by previous studies (32,33). Activation of AKT is associated with the clinical characteristics of prostate cancer. A previous study revealed that the activated AKT levels are higher in poorly differentiated prostate cancer compared with those observed in highly and moderately differentiated prostate cancer (34). The activation of AKT was positively correlated with the prognosis of prostate cancer (35). Moreover, AKT activation was correlated with prostate cancer progression and androgen independence (36). The transcriptional activity, expression and even stability of the AR are regulated by the PI3K/AKT signaling pathway (37). The inhibition of the AKT pathway by siRNA interference or by a PI3K inhibitor leads to the inactivation of the HER2/neu-activated AR signaling pathway. This process is not affected by AR expression. In addition, nicotine increases AKT phosphorylation in a PI3K-dependent manner, suggesting that smoking causes bladder cancer progression via nicotine-induced activation of the PI3K/AKT pathway (38). In addition to the action of the PI3K/AKT pathway, α5-nAChR can act via the ERK1/2 pathway, which is activated in 30% of all human cancers. It has been revealed that increased activation of ERK can inhibit apoptosis induction by endoplasmic reticulum stress in order to promote cell survival. In the present study, downregulation of α5-nAChR decreased the phosphorylated levels of the ERK1/2 protein in the DU145 and PC3 cell lines. Based on these results, it was speculated that α5-nAChR may also be a target for nicotine that could promote prostate cancer progression. However, this hypothesis requires further confirmation.

In conclusion, the data demonstrated for the first time that α5-nAChR levels were increased in prostate cancer samples and that they could promote the proliferation and migration of prostate cancer cells by the activation of the AKT signaling pathway. This finding provides clinically relevant information on utilizing α5-nAChR as a novel biomarker in order to improve prostate cancer prognosis. The present study provides a rational to develop new therapeutic approaches in order to suppress the proliferation and invasion of prostate cancer cells.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JQ designed and performed the experiments, wrote the manuscript. WYX, YPZ, CBQ, BSL, YWW, KLL, DBW and WL contributed to experimental work and data analysis. ZMZ conducted the experiments and wrote the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of the Second Hospital of Hebei Medical University. Written informed consent forms were obtained from the subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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