CHRNA7 inhibits cell invasion and metastasis of LoVo human colorectal cancer cells through PI3K/Akt signaling

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Abstract. The α7 neuronal nicotinic receptor gene (CHRNA7) is widely expressed in both the brain and periphery whereas its encoding protein of α7 neuronal acetylcholine receptor (α7nAChR) belongs to the nicotinic acetylcholine receptor family. Considerable evidence suggests that α7nAChR plays an important role in chronic inflammatory and neuropathic pain signaling and thus has been proposed as a potential target for treating cognitive deficits in patients with schizophrenia, attention deficit hyperactivity disorder (ADHD) and Alzheimer's disease. The aim of the present study was to determine the role of endogenous α7nAChR signaling in human colorectal cancer growth and metastasis. pLVX-CHRNA7 encoding the full length of CHRNA7 was constructed and transfected into LoVo human colorectal cancer cells. Cell proliferation was measured by Cell Counting Kit-8 (CCK-8), and cell migration and invasion were detected by Transwell chamber assays. Expression and activity of metastasis-related metalloproteinases (MMPs) were analyzed by western blotting and gelatin zymography, respectively. Activation of metastasis-related signaling molecules was detected by western blotting. LY294002 was used to specifically block the phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homologue (PI3K/Akt) pathway. We showed that concomitantly with an increase in α7nAChR expression after transfection, LoVo cells presented reduced abilities for migration and invasion, which was accompanied by reduced expression levels of MMP-1 and MMP-9 as well as activation of the PI3K/Akt signaling pathway. The application of LY294002 restored the migration and invasion abilities of the LoVo cells bearing CHRNA7. Collectively, we conclude that overexpression of CHRNA7 negatively controls colorectal cancer LoVo cell invasion and metastasis via PI3K/Akt pathway activation and may serve as either a diagnostic marker or a therapeutic target for colorectal cancer metastasis.

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Abbreviations: CHRNA7, α7 neuronal nicotinic receptor gene; α7nAChR, α7 nicotinic acetylcholine receptor; CRC, colorectal cancer; MMP, metalloproteinase; PI3K, phosphatidylinositol 3-kinase; Akt, v-akt murine thymoma viral oncogene homologue

Key words: CHRNA7, α7nAChR, colorectal cancer, metastasis, metalloproteinase, PI3K/Akt

Introduction

Colorectal cancer (CRC) is one of the most common malignant diseases, and the second leading cause of cancer-related mortality in both developed and developing countries (1,2). Metastasis contributes to the major cause of death for CRC and 10-25% of patients already present with liver metastasis at the time of diagnosis (3,4). Cancer metastasis is a multifactor, multistage and multistep biological process that includes cancer cell migration, adhesion, invasion, growth, neovascularization, specific organ homing and immune evasion (5). Although much progress has been achieved, the detailed molecular mechanisms involved in primary tumor cell invasion and metastasis still require further investigation.

Nicotinic acetylcholine receptors (nAChRs) are a family of integral membrane proteins responding to the binding of a neurotransmitter acetylcholine (ACh) or tobacco extracts such as nicotine. In humans, 16 different subunits of nicotinic acetylcholine receptors (α1-7, α9-10, β1-4, δ, ε, γ) are expressed on non-neuronal cells both within and outside the nervous system, which consist of five subunits forming hetero- or homo-pentamers that contain α (α1-α10), β (β1-β4), γ, δ or ε subunits (6). Genes encoding for individual nAChR subunits are named CHRNA1, CHRNA2, CHRNA3, CHRNA4, CHRNA5, CHRNA6, CHRNA7, CHRNA9 and CHRNA10 for the α subunits and CHRNB1, CHRNB2, CHRNB3,

and CHRNB4 for the β subunits. Both nAChR families are expressed in cancer cells. Accumulated evidence indicates that cancer might be triggered by altered signaling of nAChRs (7) and thus may play an important regulatory role in cancer development and progression.

α7nAChR is a special subtype consisting of five identical subunits which is expressed in many different non-neuronal cells such as vascular and brain endothelial cells, bronchial epithelial cells, keratinocytes, astrocytes, synoviocytes, thymocytes, lymphocytes, bone marrow cells, monocytes, macrophages, microglia and astrocytes (8-10) as well as cancer cells including lung, pancreatic, gastric and colon cancer (11-14). An unbalance of α7nAChR either at the gene expression or protein expression level might be involved in different diseases including Alzheimer and Parkinson disease (15). Recent studies suggest that nicotine and nicotinic derivatives could stimulate proliferation and migration of colon cancer through α7nAChRs (14,16-18). To date, it is not clear whether changes in the expression of CHRNA7 affects colorectal cancer growth and metastasis. In this study, we utilized LoVo human colorectal cancer cells to illustrate the function of CHRNA7 in controlling tumor cell growth and invasion. Based on the correlation of CHRNA7 expression with colorectal cancer metastasis, overexpression of CHRNA7, which resulted in abundant expression of α7nAChR, negatively controls LoVo colorectal cancer cell invasion and metastasis via the phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homologue (PI3K/Akt) signaling pathway and thus has potential as a therapeutic target for colorectal cancer metastasis. Thus, CHRNA7 may serve as either a diagnostic marker or a therapeutic target for colorectal cancer metastasis.

Materials and methods

Materials. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco, (Gaithersburg, MD, USA) and Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). Plasmids for pLVX and pLVX encoding the full length of acetylcholine receptor α7 gene (pLVX-CHRNA7, NM_001190455) were constructed by GenScript Corporation (Nanjing, China). The antibody against nicotinic acetylcholine receptor α7 (anti-α7nAChR) was purchased from Abcam (Oxford, UK) and antibodies against matrix metalloproteinase (MMP)-1, -2, -3, -9 and -10 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for phospho-ERK1/2, phospho-Akt (Ser473), phospho-JNK, phospho-p38, phospho-p65 (NF-κB), phospho-PI3K (p55/p85), ERK1/2, Akt, JNK, p38 and p65NF-κB were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody for β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). PI3K inhibitor LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) and goat anti-rat IgG antibody conjugated to HRP were purchased from Santa Cruz Biotechnology, Inc.

Cell culture and gene transfection. LoVo human colorectal adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC CCL-229™; Manassas, VA, USA) and were grown in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin and maintained at 37˚C in a of 5% CO_2/95% air atmosphere. Cell viability was determined by trypan blue exclusion assay. When growing at an exponential phase, the cells were transfected with pLVX-CHRNA7 or pLVX-vector using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h, the cells were harvested for subsequent experiments.

Western blotting. Cells were lysed in 1X RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (all from Cell Signaling Technology, Danvers, MA, USA). Protein concentration was determined by the Bradford protein assay. Total proteins (40 μg) were separated by SDS-PAGE and blotted onto PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated overnight on ice with the primary antibodies against α7nAChR (1:5,000), MMP-1, -2, -3, -9, -10, phospho-ERK1/2, phospho-Akt, phospho-JNK, phospho-p38, phospho-p65 (NF-kB), phosph-PI3K (55/85), ERK1/2, Akt, JNK, p38, p65NF-κB and β-actin (all dilutions 1:1,000). The next day, the membranes were washed with TBST and incubated with HRP-conjugated anti-rabbit or anti-rat secondary antibodies for 2 h at room temperature. After the final washing, the membranes were incubated with ECL reagent mixture and the signal was developed on a digital image system (FluorChem E; Proteinsimple, Santa Clara, CA, USA).

Cell proliferation assay. Cells were seeded into 96-well plates at a density of 2,000 cells per well, grown at 37˚C overnight and then transfected with pLVX-CHRNA7 or pLVX-vector. Cell growth was analyzed using a WST-8 Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) at a frequency of every 24 h until 5 days after transfection. CCK-8 solution (10 μl) was added to each well, and the cultures were incubated at 37˚C for 1 h. Absorbance at 450 nm was measured using an ultra microplate reader (SpectraMax; Molecular Devices Corp., Sunnyvale, CA, USA). Experiments were set in triplicates and repeated three times.

Transwell cell migration and invasion assay. Transwell assays was used to estimate the effect of CHRNA7 on migration and invasion of colon cancer cells. Cell invasion and migration assays were carried out using modified Boyden chambers consisting of Transwell (8-μm pore size; Corning Costar Corp., Cambridge, MA, USA) membrane filter inserts in 24-well tissue culture plates. For the invasion assay, 48 h after transfection, the cells were trypsinized and resuspended in serum-free RPMI-1640 medium at the density of $5x10^5$ cells per ml. A total of 200 μl of cells were seeded into each upper chamber of the Transwells, and 500 μl of medium with 20% FBS was added in the lower chamber. The chambers were incubated at 37˚C in a humid atmosphere with 5% CO_2. After 48 h, the non-migrating cells on the upper surface of the insert were removed with a cotton swab, and the cells that migrated to the underside of the membrane were fixed with ice-cold methanol and stained with crystal violet (0.1%). The cell surface of the insert was then photographed under an inverted microscope (Olympus CKX31; Olympus, Tokyo, Japan) for capturing images and quantified by counting the

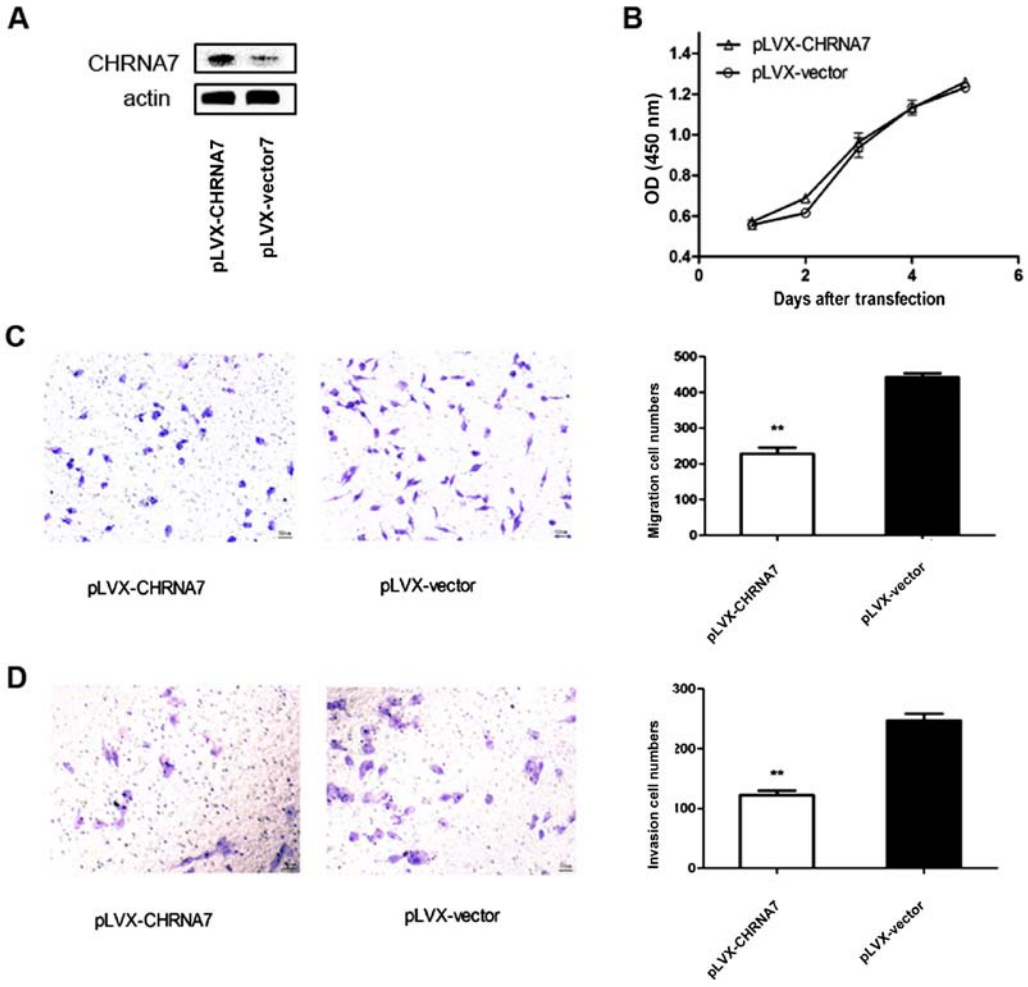

Figure 1. CHRNA7 inhibits the invasion ability of LoVo human colorectal cancer cells. (A) LoVo cells were transfected with pLVX-CHRNA7 or pLVX-vector, and the expression of CHRNA7 was confirmed by western blotting. (B) Growth of LoVo cells following transfection with pLVX-CHRNA7 or pLVX-vector was assessed by CCK-8 assay. The overexpression of CHRNA7 did not influence the proliferation of LoVo cells. (C) Overexpression of CHRNA7 led to inhibition of cell migration. Migration of the cells was calculated by counting the number of cells in five fields at x100 magnification per filter. Left, representative images, magnification x100; right, number of cells. (D) CHRNA7 suppressed the invasion of LoVo cells. **P<0.01 compared with the pLVX-vector control group. Experiments were performed in quadruplicate for at least three times.

number of cells in five fields at x100 magnification per filter. For the invasion assay, an additional 50 μl of BD Matrigel (BD Biosciences, San Jose, CA, USA) was added onto each upper chamber of the Transwells and the Transwells were placed in a 37˚C incubator for 6 h to solidify the Matrigel. The tumor cell invasion capacity was then assessed in a way similar to the migration assay.

Gelatin zymography. Gelatin zymography was used to determine the activity of MMP-2 and MMP-9. Forty-eight hours after gene transfection, cell culture supernatants of the LoVo cells were collected and concentrated in Amicon Ulta-4 Centrifugal Filter Devices (Millipore, Billerica, MA, USA). Equal amounts of total protein were then mixed with SDS loading buffer and electrophoresed on a 10% SDS polyacrylamide gel polymerized with 5 mg/ml gelatin. After electrophoresis, the gels were re-natured by soaked for 30 min at room temperature in 2.5% Triton X-100. The gels were then incubated in a developing buffer [50 mM Tris-HCl buffer (pH 7.4), 10 mM $CaCl_2$] overnight at 37˚C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 and de-stained in washing solution without dye. Gelatinolytic bands were observed as clear zones against a blue background.

Statistical analysis. All assays were performed in triplicate, and experiments were repeated at least three times. Data are presented as mean ± SE. Statistical analysis was performed using the Student's t-test to identify significant differences unless otherwise indicated. P<0.05 was considered to indicate a statistically significant difference. ImageJ software was used to quantify the results of the western blotting. GraphPad Prism 5.0 software was used to create graphics.

Results

Overexpression of CHRNA7 does not affect LoVo colorectal cancer cell proliferation. In order to evaluate the influence of CHRNA7, a plasmid of pLVX-CHRNA7 was constructed and transfected into LoVo cells. The transfection efficiency was ~80% as indicated by a GFP-expression vector (data not

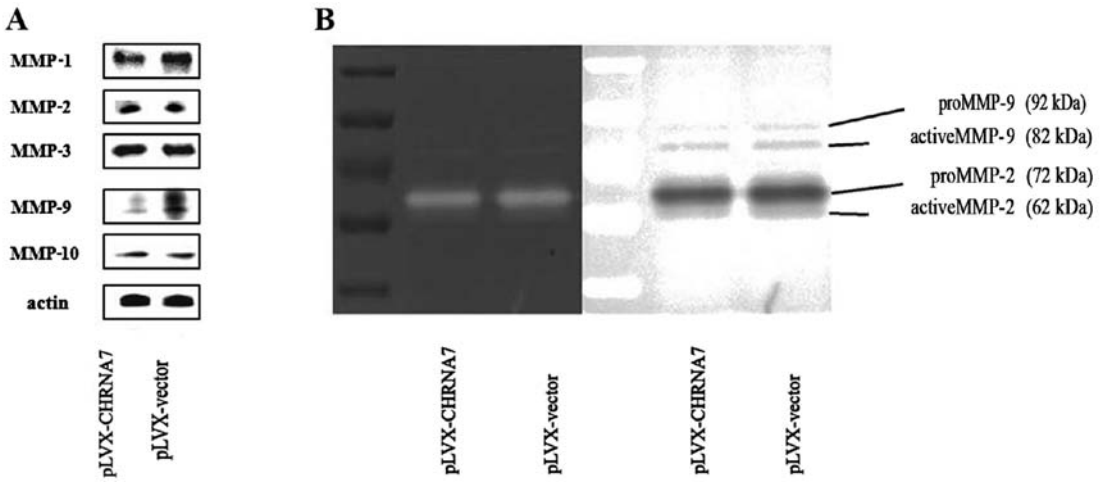

Figure 2. CHRNA7 inhibits the expression and activity of metalloproteinases (MMPs) in human colorectal cancer LoVo cells. (A) Overexpression of CHRNA7 suppressed the expression of MMP-1 and MMP-9. LoVo cell transfectants were harvested for the detection of metastasis-related gene expression by western blotting. (B) CHRNA7 reduced the MMP-9 enzymatic activity. Gelatin zymography assay was performed. Results are representative of three independent experiments.

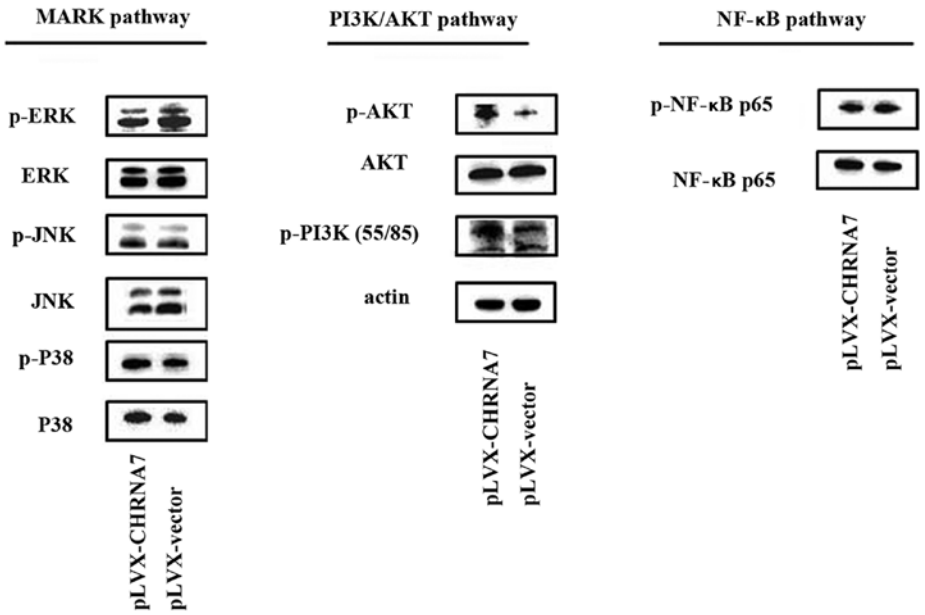

Figure 3. CHRNA7 activates the PI3K/AKT signaling pathway in colon cancer LoVo cells. After transfection, LoVo cells were harvested for protein extraction followed by western blotting. Phosphorylation of key signaling molecules representing the activation of relevant metastasis-related pathways was assessed. Antibodies against p-JNK, JNK, p-p38, p-38, p-ERK, ERK, p-NF-κBp65, NF-κBp65, p-AKT, AKT, p-PI3Kp85, p-PI3Kp55 and β-actin were used. Results are representative of three independent experiments.

shown). Western blotting revealed the abundant expression of α7nAChR in the total cell lysate of the LoVo cells 48 h after transfection (Fig. 1A).

Physiologically, we firstly measured the cell proliferation of LoVo cells with or without CHRNA7 overexpression. A CCK-8 cell proliferation assay was performed at different times after LoVo cells were transfected with pLVX-CHRNA7 or pLVX-vector. There was no difference between the two groups of cells from day 1 to 5 after transfection (Fig. 1B).

Overexpression of CHRNA7 inhibits LoVo colorectal cancer cell migration and invasion. We then detected the influence of CHRNA7 overexpression on colon cancer cell metastasis. A Transwell assay was conducted and BD Matrigel™ was used to imitate the extracellular matrix. The migratory and invasive abilities were evaluated based on the number of LoVo cells that passed through the polycarbonate membrane of the Transwell invasion chamber. As shown in Fig. 1C, the number of LoVo cells that passed through the polycarbonate membrane in the pLVX-CHRNA7 transfection group was significantly lower than that in the pLVX-vector control group ($P<0.01$). The invasion assay (Fig. 1D) showed similar results. The number of LoVo cells in the pLVX-CHRNA7 transfection group was also significantly lower than that in the pLVX-vector control group ($P<0.01$).

Overexpression of CHRNA7 inhibits MMP-1 and MMP-9 production and activity in LoVo colorectal cancer cells.

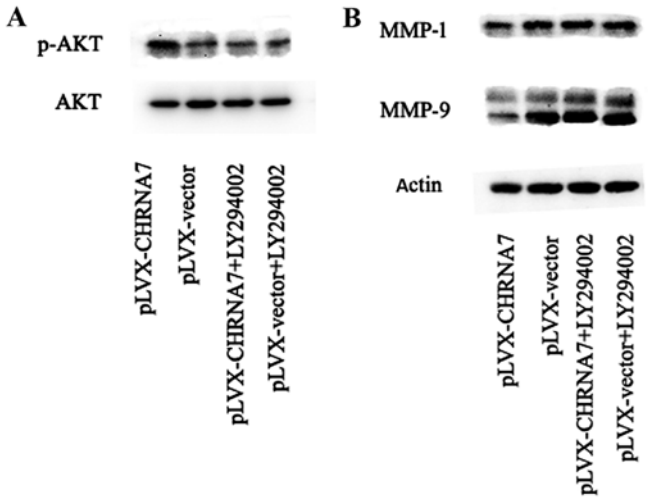

Figure 4. PI3K/AKT activation is required for the inhibitory effect of CHRNA7 on MMP expression. (A) Application of the PI3K/AKT pathway inhibitor LY294002 suppressed the phosphorylation of AKT in the CHRNA7-transfected cells. (B) LY294002 reversed the inhibitory effect of CHRNA7 on MMP-1 and MMP-9 expression. Results are representative of three independent experiments.

Next, we assessed whether the inhibition of metastatic ability by CHRNA7/LoVo was associated with the production of MMPs. Western blot analysis showed that levels of MMP-1 and MMP-9 expression in the CHRNA7-transfected cells were evidently less than these levels in the control vector-transfected cells (Fig. 2A). There was no major difference in MMP-2, -3 and -10 expression with or without CHRNA7 transfection. We also determined the enzymatic activities of the MMPs by gelatin zymography using conditioned medium. Gelatin zymography showed weaker lytic zones at the molecular masses corresponding to MMP-9 in cells with elevated CHRNA7 expression and had no effect on MMP-2 (Fig. 2B). Consistently, the activity of MMP-9 was markedly decreased when CHRNA7 was overexpressed. These data were consistent with the results observed in the cell migration and invasion assays, indicating that CHRNA7 inhibited the expression levels and activities of MMP-1 and MMP-9, and thus suppressed the invasion of colon cancer cells.

Overexpression of CHRNA7 activates PI3K/AKT in LoVo colorectal cancer cells. We then investigated the activation of relevant intracellular MAPK (JNK, p38 and ERK), NF-κB and PI3K/Akt signaling pathways (19,20) in cells overexpressing CHRNA7. Western blot assays were performed to examine the phosphorylation of JNK, p38, ERK, NF-κB (p65), Akt and PI3K (p55/p85). As detailed in Fig. 3, following comparison of the CHRNA7-transfected cells and the control vector-transfected cells, the CHRNA7-transfected cells exhibited higher phosphorylation levels of Akt and PI3K p55. The phosphorylation of ERK, JNK, P38 and NF-κB showed no difference between the CHRNA7-overexpressing cells and the control cells.

PI3K/AKT pathway inhibitor LY294002 abolishes the inhibitory effect of CHRNA7 on LoVo colorectal cancer cell migration and invasion. To determine whether enhanced PI3K/AKT activation may be involved in the inhibitory effect of CHRNA7 on the invasion of LoVo cells through Matrigel,

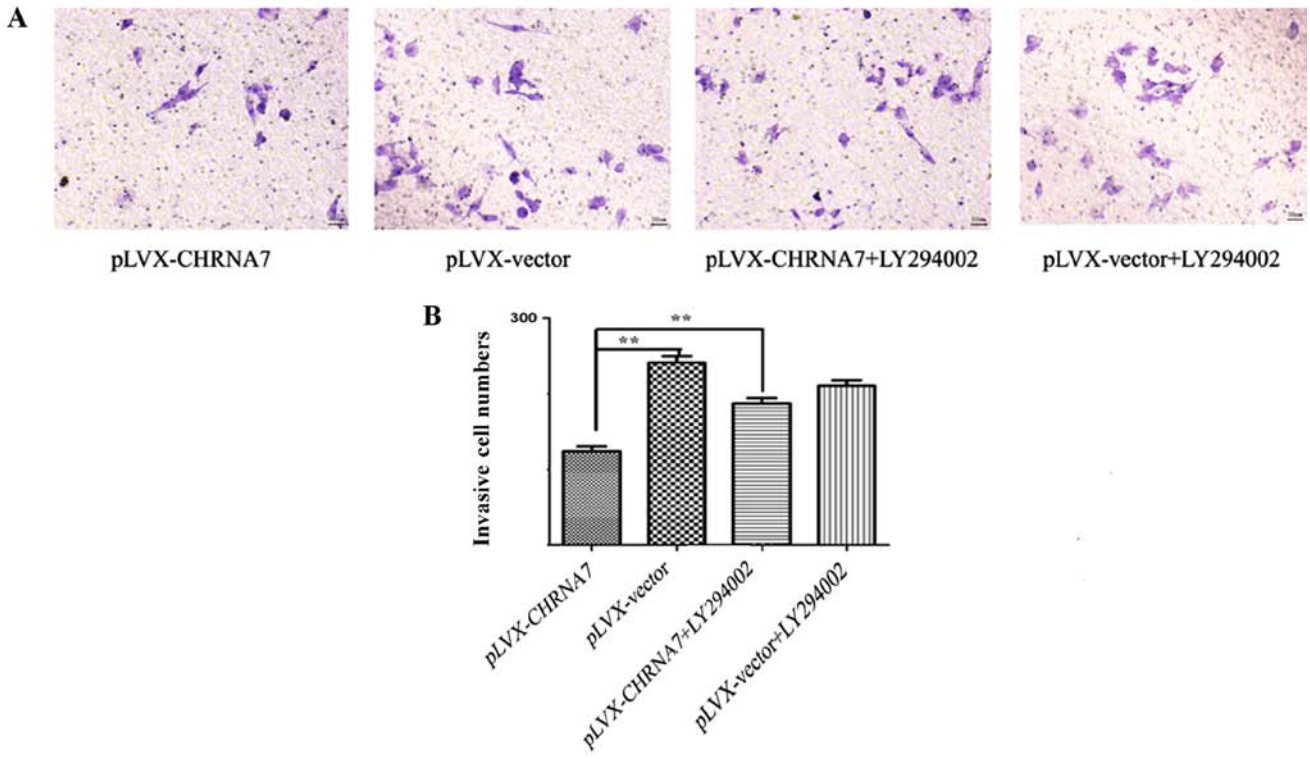

Figure 5. Inhibition of PI3K/AKT activation reverses the inhibitory effect of CHRNA7 on LoVo cell invasion. (A and B) Application of LY294002 reversed the inhibory effect of CHRNA7 on cell invasion. Results are representative of three independent experiments. **P<0.01 vs. the pLVX-vector control.

we included the PI3K/AKT pathway inhibitor LY294002 into the experimental setting. Our results indicated that LY294002 significantly suppressed the phosphorylation of AKT in the CHRNA7-transfected cells (Fig. 4A), and reversed the inhibitory effect of CHRNA7 on the expression of MMP-1 and MMP-9 (Fig. 4B). Furthermore, LY294002 significantly blocked the negative effects of CHRNA7 on cell invasion of LoVo cells (Fig. 5). Taken together, these results suggest that the CHRNA7 inhibition of the invasion of colon cancer LoVo cells and MMP expression were PI3K/AKT activation-dependent.

Discussion

The non-neuronal nAChRs have considerable implications for a number of diseases such as cancers and cardiovascular diseases. Thus, research has focused on the activation of the non-neuronal nAChR signaling pathway induced by tobacco use mimicked by nicotine binding (11,21). The nAChRs, particularly α7nAChR, can mediate nicotine-dependent upregulation of proliferative and survival genes that contribute to the growth and progression of lung (22-25) and colon cancer (14,16-18, 26). In this scenario, multiple signaling pathways are related to nAChR coupled responses to nicotine, i.e., protein kinase C (PKC) activation in breast cancer cells (28), MAPK (ERK1/2) in pancreatic carcinoma (28,29) and AKT/ERK pathways in human malignant glioma cells (30). In our previous studies, we revealed stimulation of nicotine activities of MAPK/ERK, MAPK/p38 and PI3K(p55)/Akt signaling cascades among which MAPK/p38 is responsible for the MMP-related colon cell invasion and migration (unpublished data).

In addition to nicotine and other tobacco extracts, there exists a broad range of stimuli and cell populations that may be regulated by cholinergic receptors. Vukelic *et al* showed that cholinergic neurotransmitters (ACh), similar to those released through activation of a neural reflex, regulate responses to products of the adaptive immune system, specifically immune complex (IC)-mediated activation of effector cells (31). In particular, in both lung cancer and colon cancer, ACh acts as an autocrine growth factor for cancer growth and development (32-34). Since cancer cells both produce ACh and express nAChRs, these receptors are believed to be involved in tumorigenesis, even without stimulation with nicotine. In the present study, we assessed the impact of endogenously expressed α7nAChR on colon cancer cell growth and invasion in a 'non-smoking' environment. We showed that overexpression of CHRNA7 did not affect LoVo colorectal cancer cell proliferation but inhibited cell migration and invasion. Reduced expression of MMP-1 and -9 and activation of the PI3K/Akt signaling pathway were also noted.

PI3K/Akt is recognized as a key regulator for cell biological behaviors including cell proliferation, angiogenesis, migration and invasion. It is thus believed to be involved in tumor occurrence, development (35) and metastasis (36). Although activation of PI3K/Akt is more likely associated with increased MMP production and enhanced tumor cell invasion (37,38), it is also true that activation of the PI3k/Akt pathway may be associated with reduced MMP-2 and -9 production, as a result of which the metastatic potency of colon carcinoma cells is decreased (39).

It is believed that the overall cellular response to stimulation with ACh is determined by the delicate balance between multiple signals. A switch in the predominant nAChR subtype that is expressed on the cell membrane occurs during malignant transformation, which indicates that the effects of autocrine or paracrine ACh on cancer cells might differ from the effects on non-malignant cells, even if they are situated next to each other in the same tissue (21,31). Moreover, the diversity of nAChRs could be further increased by possible variants at the genomic or mRNA level. These, together with the existence of polymorphisms in the human neuronal nicotinic acetylcholine receptor gene provide another level of variability if they influence the expression or amino acid sequence of the corresponding protein (40). In the present study, we evaluated the consequence of overexpression of CHRNA7. Our results highlight the need to dissect the physiological or pathophysiological roles of CHRNA7 and/or α7nAChR in CRC patients with or without cigarette smoking.

In summary, in the present study, we showed that overexpression of α7nAChR in human colon cancer LoVo cells, probably through the binding of cholinergic neurotransmitters i.e., ACh that are secreted by the cancer cells, activates the PI3K/AKT pathway and inhibits colon cancer metastasis and invasion. Therefore, our results indicate that CHRNA7 may serve as either a diagnostic marker or a therapeutic target for colorectal cancer metastasis. In addition, our results highlight the need for investigating the physical and pathophysiological role of CHRNA7 or α7nAChR in tumor factors other than cigarette smoking.

Acknowledgements

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