Overexpression of oxidored-nitro domain containing protein 1 induces growth inhibition and apoptosis in human prostate cancer PC3 cells

ZHONGJIE SHAN¹*, QINGLAN HOU¹*, NAN ZHANG¹, LIANG GUO¹, XINHENG ZHANG², YAOHUI MA¹ and YUDONG ZHOU¹

¹Department of Urology, The People’s Hospital of Zhengzhou, Zhengzhou, Henan 450003; ²Department of Urology, The Central Hospital of Sanmenxia, Sanmenxia, Henan 472000, P.R. China

Received June 17, 2014; Accepted July 29, 2014

DOI: 10.3892/or.2014.3407

Correspondence to: Dr Yudong Zhou, Department of Urology, The People’s Hospital of Zhengzhou, 33 Huanghe Road, Zhengzhou, Henan 450003, P.R. China
E-mail: yudongzhouhn@163.com

*Contributed equally

Key words: oxidored-nitro domain containing protein 1, prostate cancer, proliferation, apoptosis, MAPK pathway

Abstract. Previous studies have reported that oxidored-nitro domain containing protein 1 (NOR1) is a novel tumor suppressor gene identified in various types of cancer, such as nasopharyngeal carcinoma and cervical cancer. Recent studies have shown that NOR1 expression is lower in prostate cancer compared with normal prostate tissue. However, the specific function and exact mechanism of NOR1 in prostate cancer remains to be clarified. The present study aimed to investigate the function and mechanism of NOR1 in prostate cancer PC3 cells. DU145 and PC3 cells were transduced with a vector and cell viability, proliferation and apoptosis were determined. As predicted, NOR1 overexpression significantly inhibited growth and apoptosis in PC3 cells. NOR1 overexpression decreased the expression of the anti-apoptotic genes Bcl-2 and Bcl-xl and increased the level of the pro-apoptotic genes Bax and Bak in PC3 cells. Further investigation demonstrated that NOR1 overexpression activates caspase-3. Silencing of NOR1 did not inhibit growth or induce apoptosis in PC3 cells. Moreover, NOR1 inhibited proliferation and induced apoptosis via the activation of MAPK. The overexpression of NOR1 significantly inhibited tumor growth in PC3 tumor-bearing nude mice. The results suggest that the upregulated NOR1 expression was able to inhibit the progression of prostate cancer. Thus, NOR1 may be an ideal target for the treatment of prostate cancer.

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of death in males (1,2). Although substantial progress in treatment has been achieved, therapies for prostate cancer are far from satisfactory, the reason predominantly being the propensity of these tumors to metastasize. Efforts to identify new genes and signaling pathways that promote tumor progression have revealed that the oxidored-nitro domain containing protein 1 (NOR1) gene (also known as OSCP1) plays an important role in prostate cancer as well other cancer types (3-5). Evidence suggests that the expression of NOR1 is significantly decreased in prostate cancer cells (3); however, the specific function of NOR1 in the progression of prostate cancer remains to be elucidated.

NOR1, a novel tumor-suppressor gene, was first isolated from a nasopharyngeal carcinoma (6). The human NOR1 gene (chromosome 1 open reading frame 102) is located on 1p34.3 (7) and has nine introns and 10 exons according to the human genome working sequence (8). Moreover, data from the PROSITE database suggest that the NOR1 gene has four N-myristoylation sites, two tyrosine phosphorylation sites and cAMP and cGMP-dependent protein kinase phosphorylation sites (8). NOR1 is expressed in many tissues and cells, and more recently, it was shown to restrict expression in the testis and neurons. Thus, this gene has been identified as a potential cancer/testis/brain antigen (3). NOR1 is usually highly expressed in many tissues, such as brain, testis, the upper respiratory tract (such as the nasopharynx), but more weakly expressed in many types of cancer tissues, such as nasopharyngeal carcinoma, lung, ovarian, vulvar, kidney, leukemia, uterus, testis, thyroid gland, cervical and prostate (5,7,9,10).

Cancer cell proliferation and apoptosis are important in tumor progression. Reagents or molecules that can inhibit cancer cell proliferation and induce apoptosis may suppress tumor progression. Apoptosis, a form of programmed cell death, is regulated by the Bcl-2 family and caspase family of proteins (11). Evidence suggests that genes, such as p53 (12,13), caspase-3 (14), caspase-9 (13), Bcl-2 (15), Bcl-xl (15) and Bax (16) regulate cell apoptosis. Ouyang and colleagues (5) found that the overexpression of NOR1 inhibits human nasopharyngeal carcinoma and cervical cancer cell proliferation and induces apoptosis. Authors of that group also showed that NOR1 overexpression can activate caspase-9, increase pro-apoptotic Bax and p53 expression and decrease...
anti-apoptotic Bcl-2 expression (5). Although NOR1 has many positive effects in many types of cancer, the function and signaling mechanism of NOR1 overexpression in prostate cancer cells has not been defined. Therefore, it was hypothesized that NOR1 overexpression may inhibit the growth of prostate cancer cells and induce cell apoptosis. In the present study, we demonstrated that NOR1 overexpression activates caspase-3 and -9 and increases the expression of Bax and Bak, while decreasing the level of Bcl-2 and Bcl-xl in DU145 and PC3 prostate cancer cells. To the best of our knowledge, the study provides the first direct evidence in prostate cancer cells that NOR1 overexpression can lead to cell growth inhibition and apoptosis induction by altering the expression of apoptosis-related genes through the MAPK signaling pathway.

Materials and methods

Animals. Procedures were carried out according to the protocols approved by the Ethics Committee for Animal Experimentation of the People's Hospital of Zhengzhou (Zhengzhou, China). All efforts were made to minimize the suffering of the animals. MF-1 nude mice (6-8 weeks old) were obtained from the Animal Center of Zhengzhou University, Zhengzhou, China.

Antibodies. Rabbit Anti-NOR1 polyclonal antibody, and mouse anti-caspase-3, mouse anti-Bcl-2, mouse anti-Bcl-xl, mouse anti-Bax, mouse anti-Bak, and mouse anti-β-actin monoclonal antibodies, as well as HRP-conjugated rabbit anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were purchased from Abcam (Cambridge, MA, USA).

Cell culture. The DU145 (HTB-4) and PC3 human prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin-streptomycin and 1% glutamine at 37°C under a humidified atmosphere of 5% CO₂.

Clone and expression vectors construction of NOR1. pcDNA3.1-myc-his-NOR1 was constructed as previously described (5). Briefly, total RNA was extracted from the DU145 and PC3 prostate cancer cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (2 µg) was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Shiga, Japan). The cDNAs were used for the amplification of NOR1 using two primers (17): sense 5'-GTGCCTGCTGGTCACCTT TCAA-3' and antisense 5'-GGATACCTCGGATGTGCA GT-3'. pcDNA3.1-myc-his-NOR1 was generated using the ViraPower Adenoviral Expression System (Invitrogen) according to the operating protocols. The transfection experiment was performed with pcDNA3.1-myc-his-NOR1 (NOR1) or the control plasmid (control) using Lipofectamine 2000 according to the manufacturer's instructions.

Cell viability assay. The viability of the DU145 and PC3 human prostate cancer cell lines was determined using the trypan blue exclusion assay, as previously described (18). Briefly, transfected and control cells were seeded in 96-well plates at 1x10⁴ cells/well and cultured in RPMI-1640 for different periods of time (0, 24, 48, 72 and 96 h). Trypan blue (40 µl; Sigma, St. Louis, MO, USA) was added to cell suspensions (10 µl) in PBS. The cells were then washed three times with DMSO, followed by counting under a light microscope.

MTT proliferation assay. DU145 and PC3 cell proliferation was detected by the previously described 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (19). Briefly, the cells were transfected as described above, and then plated in 96-well plates. The cells were cultured for 72 h, then 20 µl MTT (5 mg/ml) was added to the cancer cells for 4 h at 37°C. DMSO (200 µl) was added to solubilize the crystals at room temperature. The optical density (OD) was measured at 490 nm by a spectrophotometer (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA). Data were calculated using averaged results.

Detection of apoptotic cells by flow cytometry. To analyze apoptotic PC3 cells by Annexin V-FITC and propidium iodide (PI) staining by flow cytometry (Beckman-Coulter, Brea, CA, USA), 1x10⁴ cells/well were plated in 96-well plates. The cells were trypsinized and harvested by centrifugation. Furthermore, the cells were incubated with Annexin V and PI for 20 min at room temperature. Apoptotic cells were then analyzed by flow cytometry (Beckton-Dickinson, Franklin Lakes, NJ, USA).

Nuclear morphological observation of PC3 cancer cells. PC3 cancer cells were plated in 96-well plates at 1x10⁵ cells/well. The cells were fixed using 4% formaldehyde, and washed twice with PBS. Subsequently, the cells were stained with Hoechst 33258 staining solution using the Hoechst Staining kit according to the manufacturer's instructions (20,21). Stained nuclei of PC3 cells were observed under a reflected fluorescence microscope (Nikon MF30 LED; Nikon, Tokyo, Japan).

Total RNA extraction and quantitative reverse transcription-PCR. NOR1 mRNA levels were measured in the PC3 prostate cancer cell line according to a previously described method, with minor modifications (22). Total RNA was isolated from cells using TRIzol, according to the manufacturer's instructions. RNA was reverse transcribed using a high capacity RNA-to-cDNA kit (Applied Biosystems). RT-qPCR was performed using an ABI PRISM 7000 Sequence Detection system (Applied Biosystems) with SYBR-Green ReadyMix (Applied Biosystems). The primer sequences used for RT-qPCR were: NOR1 forward 5'-ACCTGCACATCCGAGTCC-3' and reverse 5'-CTGGCCAAGAAATTCAGCTC-3'; β-actin forward 5'-TCCCTGGAAGAGTAGCTACA-3' and reverse 5'-AGGAAGGAGCTGGAAGAG-3'. The primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Expression levels of the relative genes were calculated using the 2^-∆∆CT method (23). β-actin mRNA was used as an internal control.

Western blot assay. The protein expression in PC3 cells was determined according to a previously described method, with
To obtain whole-cell lysates, the cells were resuspended in ice-cold lysis buffer [1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4)]. The protein concentration in the lysates was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Nantong, China). The proteins (40 µg/lane) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk, the membranes were probed with mouse monoclonal and rabbit polyclonal antibodies followed by HRP-conjugated rabbit anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG. Bound proteins were detected using the ECL reagent (Boehringer Mannheim, Mannheim, Germany).

Small interfering RNA transfection. PC3 prostate cancer cells with the NOR1 protein were transfected with NOR1 siRNA (siNOR1) or the control siRNA (siMock) using Lipofectamine 2000 (Invitrogen). The NOR1 siRNA oligonucleotides used were: 5'-GGCCAGCATGGATAAGCTCTA-3'; and siRNA scrambled: 5'-GGATAAAGCGTGCCCGTACTA-3'. Stable transfectants of PC3 cells were selected with culture medium containing puromycin (Invitrogen). Viable colonies were picked up and transferred to new dishes 2-3 weeks after transfection. siNOR1- and siMock-transfected cells were used for subsequent experiments.

Prostate cancer model. Male MF-1 nude mice (6-8 weeks old) were housed under pathogen-free conditions. PC-3 cancer cells (1x10^6) infected with a control or NOR1-overproducing vector were implanted subcutaneously in 6- to 8-week-old male nude mice, respectively. Tumor growth was monitored regularly using calipers. The tumor volume was calculated as follows: (larger diameter) x (smaller diameter) x (width) (25).

Statistical analysis. Data are expressed as means ± SEM. The differences between the control and experimental groups were compared by the Dunnett's test. P<0.05 was considered statistically significant. All experiments were repeated at least three times.

Results

Increased NOR1 levels in DU145 and PC3 prostate cancer cell lines transduced with pcDNA3.1-myc-his-NOR1. To overexpress NOR1 in the DU145 and PC3 prostate cancer cell lines, we used a eukaryotic expression vector, pcDNA3.1-myc-his, which encodes NOR1 under normal conditions. The expression of NOR1 in prostate cancer cells was determined by RT-qPCR and western blot analysis. The expression of NOR1 in prostate cancer cells was determined by RT-qPCR and western blot analysis. The RT-qPCR results (Fig. 1A) showed that NOR1 mRNA levels in DU145 and PC3 cells transduced with pcDNA3.1-myc-his-NOR1 for 72 h were much higher than in the control group. The protein results indicated that NOR1 protein levels were higher in DU145 and PC3 cells transduced with pcDNA3.1-myc-his-NOR1 for 72 h (Fig. 1B). The mRNA and protein expression was also upregulated in DU145 and PC3 transduced with pcDNA3.1-myc-his-NOR1 for 24, 48 and 96 h (data not shown).

Effect of NOR1 overexpression on DU145 and PC3 cell viability and proliferation. The cytotoxic effect of NOR1 overexpression on DU145 and PC3 cells was determined by the trypan blue exclusion assay. The results showed that the viability of DU145 and PC3 cells was significantly reduced after NOR1 overexpression in a time-dependent manner (Fig. 2A). We also measured the effect of NOR1 overexpression on DU145 and PC3 cell proliferation using an MTT assay. As shown in Fig. 2B, there was a marked decrease in the proliferation of DU145 and PC3 cells with NOR1 overexpression. We found that NOR1 overexpression led to an almost 49 and 53% decrease in DU145 and PC3 cell numbers, respectively. This finding indicated a positive effect of NOR1 on the inhibition of proliferation and survival of DU145 and PC3 cell. Since PC3 cells were more sensitive to NOR1 overexpression, this cell line was selected for subsequent experiments.

Effect of NOR1 overexpression on PC3 cell apoptosis. Evidence suggests that NOR1 overexpression can induce human nasopharyngeal carcinoma and cervical cancer cell apoptosis (5). Thus, we tested whether the change in PC3 cell numbers in our experiments was also mediated by NOR1-induced apoptosis. Apoptosis was first measured by the flow cytometric analysis of PC3 cells double-stained with Annexin V and PI.
The results suggested that NOR1 overexpression induced cell apoptosis, and the results showed a significant increase in the percentage of early (Annexin V-positive and PI-negative) and late (Annexin V-negative and PI-positive) apoptosis. The total apoptotic rate increased by 46% when cells were transduced with the NOR1 vector for 72 h (Fig. 3A and B).

To confirm that NOR1 overexpression induces apoptosis in PC3 cells, an additional apoptotic marker, Hoechst 33258 staining, was evaluated (26). Membrane-permeable Hoechst 33258 is a blue fluorescent dye that stains the cell nucleus (27). Thus, we measured the changes in cell nuclear morphology by Hoechst staining using fluorescence microscopy. As shown in Fig. 3C, the control and vector groups of PC3 cells showed uniformly light blue nuclei under the fluorescent microscope. The NOR1 cells showed smaller nuclei and condensed staining because of karyopyknosis and chromatin condensation.

Given the profound roles of Bcl-2 and the caspase family in triggering apoptosis, the anti-apoptotic proteins Bcl-2 and Bcl-xl and the pro-apoptotic proteins Bak and Bak, as well as the activity of caspase-3 in NOR1 overexpression-mediated apoptosis in PC3 cells were evaluated. The western blot results revealed that the expression of Bcl-2 and Bcl-xl was decreased, whereas the expression of Bak and Bak was simultaneously upregulated (Fig. 3D; P<0.05). Moreover, NOR1 overexpression showed a marked increase in the activity of caspase-3 (Fig. 3E; P<0.05). Thus, it appears that, in the PC3 prostate cancer cell line, apoptosis was induced by NOR1 overexpression through a caspase-dependent pathway.

Specific inhibition of NOR1 mRNA and protein expression by NOR1-specific siRNA. The above results were designed to reveal the main effects of overexpressing NOR1 in prostate cancer cells. To detect the effects of inhibiting the expression of NOR1 in NOR1-overexpressing cells, a complementary approach was employed using the RNA interference method. PC3 cells were transfected with NOR1 siRNA (siNOR1) or control siRNA (siMock) and analyzed after 48 h of growth. The RT-qPCR and western blot results showed that the level of NOR1 in cells transfected with siNOR1 was significantly decreased (Fig. 4A and B). The effect of NOR1 silencing on cell proliferation and apoptosis was determined. The results shown in Fig. 4C indicate that there was a significant increase in PC3 cell growth in the siNOR1-transfected group compared with the siMock-transfected group. Moreover, cell apoptosis was also markedly decreased in the siNOR1-transfected group (Fig. 4D and E). These results showed that growth inhibition and apoptosis in PC3 cells is associated with the overexpression of NOR1.

Overexpression of NOR1 is directly related to the activation of MAPK. An important role for the p38 MAPK pathway in
apoptosis had been described in many cell types, including prostate cancer cells (28-30). Moreover, Gui et al (8) found that the NOR1 gene can activate the MAPK signaling pathway. Therefore, to determine a potential mechanism that might be involved in the pro-apoptotic and growth inhibition effect of NOR1 in prostate cancer cells, the effect of NOR1 overexpression on the level of MAPK and phosphorylated MAPK (pMAPK) was assessed. The results showed that pMAPK was significantly increased in NOR1-overexpressing cells and decreased in siNOR1-transfected cells (Fig. 5). This result showed that the MAPK pathway is inhibited in siNOR1-transfected cells.

To confirm whether MAPK mediates NOR1 overexpression-induced cell apoptosis, cells were treated with the MAPK inhibitor PD98059. As shown in Fig. 5, PD98059 inhibited NOR1 overexpression-induced MAPK activation. Apoptosis and proliferation in PC3 cells were determined and the results showed a marked increase in PC3 cell growth and a significant decrease in apoptosis in cells treated with PD98059 compared with the NOR1-overexpressing group (Fig. 4D).
Effect of NOR1 overexpression on prostate cancer growth. To examine whether NOR1 overexpression would induce efficient antitumor effects, normal PC3 cells, control transfectants (vector) and NOR1-overproducing cells (vector+NOR1) were injected into the flanks of nude mice, and the tumor size was measured at frequent intervals (Fig. 6). The results showed that NOR1 overexpression significantly inhibited tumor growth in vivo. This result showed that NOR1 overexpression induces a marked antitumor effect.

Discussion

The main findings of the present study demonstrate that: i) NOR1 overexpression can induce growth inhibition and apoptosis in prostate cancer cells; ii) the anti-apoptotic effect of NOR1 is apparently involved in regulating the expression of apoptosis-related genes, i.e., caspase-3, Bcl-2, Bcl-xl, Bax and Bak; iii) NOR1 silencing has no apparent effect on PC3 cell growth and apoptosis; iv) NOR1 overexpression significantly inhibited tumor growth in vivo. This result showed that NOR1 overexpression induces a marked antitumor effect.
be explained by growth inhibition and pro-apoptotic effects of NOR1 in prostate cancer cells.

In the present study, we analyzed the effects of NOR1 overexpression on prostate cancer cell proliferation and apoptosis. The human NOR1 gene, located on 1p34.3 (7), is a novel tumor-suppressor gene. Evidence suggests that the NOR1 promoter region is always methylated in nasopharyngeal carcinoma and leukemia, leading to lower NOR1 expression in these cancer cells (4,7). Moreover, some studies suggest that NOR1 is downregulated in prostate cancer cells compared with normal prostate tissue (5). To determine the antitumor effects of NOR1, we constructed a recombinant expression vector (pcDNA3.1-myc-his-NOR1) and transfected the vector into DU145 and PC3 cells. The RT-qPCR and western blot results indicate that NOR1 expression is significantly upregulated in these two types of cells (Fig. 1). In the present study, the overexpression of NOR1 in prostate cancer cells induced cell growth inhibition and apoptosis. We found that a high level of NOR1 was associated with a decrease in cell proliferation and an increase in cell apoptosis (Figs. 2 and 3). Simultaneously, we found that NOR1 siRNA increased cell survival and decreased apoptosis (Fig. 4). These data suggest that the upregulation of NOR1 expression was able to inhibit the progression of prostate cancer.

Apoptosis, which is also known as programmed cell death, is an evolutionarily conserved cell suicide process executed by caspases and regulated by the Bcl-2 protein family (31,32). In the present study, we found that NOR1 overexpression can downregulate the anti-apoptotic genes Bcl-2 and Bcl-xL and upregulate the pro-apoptotic genes Bax and Bak (Fig. 3). Moreover, we found that NOR1 overexpression induced a marked increase in the activity of caspase-3 (Fig. 3). Therefore, these results show that NOR1 overexpression-induced PC3 prostate cancer cell line apoptosis is dependent on the caspase pathway. Further investigation suggested that the mechanism of NOR1 overexpression-induced growth inhibition and apoptosis occurred through the MAPK signaling pathway. NOR1 overexpression upregulated the level of pMAPK, whereas suppression of the expression of NOR1 inhibited the phosphorylation of MAPK (Fig. 5). These results show that the expression of NOR1 activated the MAPK signaling pathway. The present study demonstrates that the MAPK inhibitor PD98059 attenuated the action of NOR1 overexpression-induced cell growth inhibition and apoptosis (Fig. 4). We also found that NOR1 overexpression induced strong antitumor effects in a prostate cancer model.

In summary, our findings have shown that the overexpression of NOR1 contributes to the inhibition of prostate cancer. Overexpression of NOR1 inhibits the proliferation of prostate cancer cells and promotes cell apoptosis. Furthermore, our study suggests that the activity of caspase-3, and the expression of Bcl-2, Bcl-xL, Bax and Bak are also regulated by NOR1, thus, modulating apoptosis in PC3 cells. The present study also suggests that NOR1 modulates the proliferation and apoptosis of PC3 cells through the MAPK signaling pathway. Additionally, NOR1 overexpression induced strong antitumor effects in a prostate cancer model. These findings support the hypothesis that increasing NOR1 expression may be an ideal therapeutic approach for the treatment of prostate cancer.

References