

**MOLECULAR CLONING AND ANALYSIS OF THE HUMAN *PCANI*
(*GDEP*) PROMOTER**

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Abstract: Human *PCANI* (prostate cancer gene 1) is a prostate-specific gene that is highly expressed in prostate epithelial tissue, and frequently mutated in prostate tumors. To better understand the regulation of the *PCANI* gene, a 2.6-kb fragment of its 5' flanking region was obtained by PCR. Its promoter activity was examined via the dual-luciferase reporter assay after it had been cloned into a pGL₃-basic vector generating pGL₃-p2.6kb and transfected into LNCaP cells. pGL₃-basic and pGL₃-control were respectively used as the negative and positive controls. Sequence analysis with the MatInspector database showed that some possible binding sites for the transcriptional factors, NKX3.1, P53, SP1, cEBP and the PPAR/RXR heterodimers may locate on a 2.6-kb region upstream of the *PCANI* gene. To examine the relevant regulation of *PCANI*, pGL₃-p2.6kb was transfected into the prostate cancer cell line LNCaP, which was treated with R1881 (10^{-7} ~ 10^{-9} mol/l), 17 β -estradiol (17 β -E₂, 10^{-7} ~ 10^{-9} mol/l), all-trans-retinoic acid (all-trans-RA, 10^{-5} ~ 10^{-7} mol/l) or 9-cis-retinoic acid (9-cis-RA, 10^{-5} ~ 10^{-7} mol/l), and eukaryotic expression plasmids of *NKX3.1*, *p53*, *Sp1*, *Pten*, *PPAR γ* or *cEBP α* were cotransfected with pGL₃-p2.6kb into LNCaP cells. pRL-TK, a Renilla luciferase reporter vector, was cotransfected into all the transfection lines as an internal control. The

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Abbreviations used: ATCC – American type culture collection; ARE – androgen response element; cEBP – CCAAT/enhancer binding protein; ERE – estrogen response element; *GDEP* – gene differentially expressed in prostate; GRE – glucocorticosteroid response element; LOH – loss of heterozygosity; *PCANI* – prostate cancer gene 1; PPAR/RXR – peroxisome proliferators activated receptor/retinoic acid receptor; Pten – phosphatase and tensin homologue deleted on chromosome ten; RA – retinoic acid

activities of pGL₃-p2.6kb (*PCANI* promoter) were analyzed via the dual-luciferase reporter assay 48 h after transfection. The results showed that 9-cis-RA enhanced the *PCANI* promoter activity in a dose-dependent manner, while R1881, 17 β -E₂ and all-trans-RA had no significant effect on *PCANI* promoter activities. Cotransfection with pGL₃-p2.6kb and the expression plasmids of *NKX3.1*, *p53*, *Sp1* or *Pten* respectively resulted in 1.66-, 2.48-, 2.00- and 1.72-fold 2.6 kb *PCANI* promoter activity increases relative to the controls, which were cotransfected with pcDNA3.1(+), while cotransfection of *PPAR γ* and *cEBP α* yielded no significant effect on *PCANI* promoter activities. These results could be applied for further study of the function and transcription regulation of the *PCANI* gene in prostate development and carcinogenesis.

Key words: *PCANI*, Promoter, Transfection, Luciferase reporter assay, Prostate cancer cell

INTRODUCTION

Prostate cancer is the most frequently diagnosed neoplasia in men and one of the leading causes of cancer-related deaths in men over 60 [1]. Once prostate cancer is identified in an individual, there is a limited number of treatment options. With no effective cure for the disease after it metastasizes and becomes androgen-independent [2, 3], it is important to determine its biology.

PCANI (prostate cancer gene 1, also known as *GDEP*) is highly expressed in the prostate epithelial tissue, and frequently mutated in prostate tumors [4-6]. It is localized to chromosome 4q21, a region of the genome that experiences frequent loss of heterozygosity (LOH) in prostate cancer. It is mutated in 35% of tumor samples. *PCANI* gene expression is localized to the prostate epithelial cells with the highest expression in the basal epithelial cells and lesser expression in the acinar epithelial cells [6]. This gradient of expression is lost in prostate tumors, where diffuse expression is observed throughout the tumor [6].

To elucidate the regulation of the *PCANI* gene, a 2.6-kb fragment of its 5' flanking region was amplified by PCR and cloned into the multiple clone sites of pGL₃-basic, a promoter-less luciferase reporter vector, so that its promoter activity could be analyzed via the dual-luciferase report assay. This research will provide insight into the regulatory mechanism of *PCANI* expression in further studies.

MATERIALS AND METHODS

Amplification and subcloning of a 2.6-kb fragment of the 5' flanking region of the *PCANI* gene

Human genomic DNA was extracted from white blood cells using the method of rapid isolation of mammalian DNA. The primer pair PF (5'-cccTAGCTAgccatctctgcagctctgac-3'; with a *Nhe* I site at its 5' end) and PR (5'-cccAAGCTTcgctctgactctcttc-3'; with a *Hind* III site at its 5' end) were

used to amplify the 5' flanking region of the *PCAN1* gene from the extracted human genomic DNA. The PCR was conducted at 96°C for 2 min followed by 32 cycles at 98°C for 20 s, and 68°C for 10 min. The PCR-amplified fragment was about 2.6 kb (+32 bp to -2598 bp), and it was subcloned into a T/A clone vector of pMD18-T (TaKaRa Biotech Co, Dalian China) to form a T/A cloning recombinant (pMD18-2.6 kb).

Construction of luciferase reporter plasmid

The 2.6 kb fragment was excised from pMD18-2.6 kb with *Nhe* I and *Hind* III (TaKaRa), and ligated into the equivalent site of the pGL₃-basic vector (Promega, Madison WI, USA) to form the *PCAN1* promoter-luciferase reporter constructs, designated pGL₃-p2.6kb. The resulting construct was confirmed by restriction enzyme digestion and sequence analysis using the general primers Rvprimer3 and Rvprimer2.

Cell culture

LNCaP cells (ATCC – American Type Culture Collection) were grown at 37°C in 5% CO₂ with RPMI 1640 (Gibco, BRL Gaithersburg, MD, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml ampicillin and 100 U/ml streptomycin. Within 60 h of passage, LNCaP cells with more than 90% confluency were used for transfection.

Transient transfection

LNCaP cells were transfected with lipofectimineTM 2000 (Promega) in 24-well plates. Each well contained 1.5 x 10⁵ cells, 1.0 µg pGL₃-p2.6kb, 0.04 µg of the internal control vector pRL-TK, 2 ml lipofectimineTM 2000, and 500 µl RPMI 1640 medium without serum or antibiotics, along with 0.5 µg of one of the eukaryotic expression plasmids pcDNA3.1-*NKX3.1* (constructed in our lab), pCMV-*p53* (Clontech, Palo Alto, CA), pcDNA3.1-*Sp1*, pcDNA3.1-*Pten* (from Dr. Young, Mayo clinic, USA), pcDNA3.1-*PPARγ*, or pcDNA3.1-*cEBPα* (constructed in our lab). All the cells underwent the dual-luciferase reporter assay 48 h after the completion of the transfection procedure, following the protocol recommended by Promega.

Treatment of the transfected LNCaP cells

The stocks of R1881, 17β-E₂, all-trans-RA and 9-cis-RA (Sigma, St.Louis, MO, USA) were prepared in ethanol. After the transfection of pGL₃-p2.6kb in 24-well plates, the LNCaP cells were treated for 24 h with R1881 (10⁻⁷~10⁻⁹ mol/l in 2% charcoal treated FBS- RPMI 1640 media), 17β-E₂ (10⁻⁷~10⁻⁹ mol/l in 2% charcoal treated FBS- RPMI 1640 media), all-trans-RA (10⁻⁷~10⁻⁹ mol/l in 10% FBS-RPMI 1640 media), or 9-cis-RA (10⁻⁵~10⁻⁷ mol/l in 10% FBS-RPMI 1640 media). The controls received the ethanol vehicle at a concentration equal to that for the treated cells. All the cells underwent the dual-luciferase reporter assay 48 h after the completion of the transfection procedure.

Dual-luciferase reporter assay

The activities of firefly luciferase in pGL₃ and Renilla luciferase in pRL-TK (Promega) were determined following the dual-luciferase reporter assay protocol recommended by Promega. The cells were rinsed with PBS after harvest, and cell lysates were prepared by manually scraping the cells from the culture plates in the presence of 1 x PLB (passive lysis buffer). 20 µl of cell lysate was transferred into luminometer tubes containing 100 µl LAR. Firefly luciferase activity (M₁) was measured first, and then Renilla luciferase activity (M₂) was measured after the addition of 100 µl of Stop&Glo Reagent.

RESULTS

The construction and identification of the *PCANI* promoter-luciferase reporter plasmid (pGL₃-p2.6kb)

pGL₃-p2.6kb was constructed with pGL₃-basic and the 2.6-kb fragment that was excised from pMD18-2.6 kb with *Nhe* I and *Hind* III. The pGL₃-p2.6kb construct was confirmed by restriction enzyme digestion (Fig. 1) and DNA sequencing (Fig. 2). Analyses of the 2.6-kb sequence using MatInspector 2.2 (<http://www.gene-regulation.com>) revealed potential binding sites for some important transcriptional factors within the 2.6-kb sequence, as shown in Fig. 2.

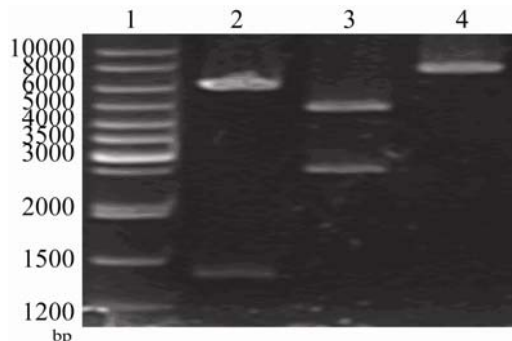


Fig. 1. Identification of pGL₃-p2.6kb by restriction enzyme digestion and electrophoresis. Lane 1: DNA marker, lane 2: pGL₃-p2.6kb cut by *Sac* I, producing 1.4-kb and 6-kb fragments, lane 3: pGL₃-p2.6kb cut by *Nhe* I and *Hind* III, producing a 2.6-kb insert and a 4.8-kb vector, lane 4: pGL₃-p2.6kb opened by *Hind* III, producing a 7.4-kb fragment.

The transient transfection and promoter activity assay of pGL₃-p2.6kb

The firefly luciferase expression driven by the 2.6-kb *PCANI* promoter was examined to evaluate its promoter activity. The transfections of the pGL₃-control and pGL₃-basic were respectively used as the positive and negative controls, and the pGL₃-promoter containing a SV40 promoter was used for the comparison of promoter activity with the 2.6-kb *PCANI* promoter. The dual-luciferase reporter assay (M1/M2) yielded a result of 0.49 after 48 h of pGL₃-p2.6kb transfection, which was about 60% of the pGL₃-promoter (SV40 promoter)

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-2598 catctctgca gtctcgacca taagaaacgg ccacgcccag ggggctgttt
ataagcctat acctccaggt gcacattctc cttctcaggg atgttccttg ctgagaaaaa
gaattcagca atgtttctcc catttgcttt tgaagaaga gaaatatggc tctgttctgc
cEBP binding site(0.96)
ccggctcact ggcagtcaga gttaaagggt atctctctta ttccttgaac aattgctgtt
atcctgttct ttttcaagg tgctcagttt tcatattgct caaacacaca tgctgtacaa
cEBP binding site( 0.940)
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caggattaag agattaaagt aaagacagge ataggaatc acaagggtat tgattgggga
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tccacattct tctgcatgg cttcagctgg tccctcgtt tggggtccc gacttcccac
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ccgattttga tgcaggattc tttgactggt ctggcacact aaaacaage cgattaaaca
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NKX3.1 binding site(0.878)
ggggtttaat ccataaagat tttctgccat ctgatctaac gcctcagctc caggcaaat
ggataagtga gcttgagagg ctcaaaaaat ttgtttcttt aatttagtta tgcctaatga
taaattatct tctctacca gaagatgtcc tttgaccatt tccatgaat gatcgtctc
PPAR/RXR binding site (0.807)
attataggaa tatggggtga ttagaaatc tgaagtattc caatcgcact gcatttgcac
gagatgctcg agactcacta cccgatctcc aaccacaata acagactgtc ttaaatcatt
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tcttgagatc aaaaatgggag ttaatacaag tgcatagatg acaattaatg cattggacag
PPAR/RXR binding site (0.884)
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agctctgtat gggaaatagtc aggttggagg taagtaaagc agaatgtctg ggtctacatt
NKX3.1 binding site(0.879)
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cagtcagac atggcaatag ccaatttcca aagttctggg tgttctgggc tcagaatggg
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aataaaaataa gtagcctcca ggcattecc tccaccagag gagcaattgt tttttaaata
NKX3.1 binding site(0.900)
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cEBP binding site(0.940)
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cggttttctt gcaggtttat atggaaagta tgggtatctc ccattactcc tcctttcatt
tgttttaaag gagaaagga gaggccagag accaaatgct tcattttate tgtatggaa
PPAR/RXR binding site (0.718)
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p53 binding site (0.934)
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cctgggttgg cagggcaaca gttatctgtg gctccaggca tccacacact attgttagtg
Spl/Sp2 binding site(0.861)
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taagcccaat aagaatattt atgtgtagca ggtaaatcag tgtgagagga aactgggtgag
acagaaagta taaggaggag aatcattaa taaaacttat tgtaaagtgag attctgaaga
NKX3.1 binding site (0.885)
aggaagagaa gaacagtgtt aagtatcctg gcaaaggagc attagggtta cagatagaat
NKX3.1 binding site(0.916)
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caatgtaatc aggaagggcc tttaaagtGa gagagggagg cagaagagga agtcagagcg
+1

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Fig. 2. The sequence of the 2.6-kb fragment of the 5' flanking region of the *PCAN1* gene. The 2.6-kb fragment (from +32 to -2598) of the 5' flanking region of the *PCAN1* gene was inserted into the pGL₃-basic vector to form the *PCAN1* promoter-luciferase reporter constructs designated pGL₃-p2.6kb. The sequence of the 2.6-kb fragment was confirmed by bidirectional sequence analysis using the general primers Rvprimer3 and Rvprimer2. The sequence did not reveal a clear TATA-box in the promoter region. Analyses of the 2.6-kb sequence using MatInspector 2.2 revealed potential binding sites for some important transcriptional factors, which are shown in the underlined sequences. The numbers in the brackets represent the Matrix similarity of the binding sites. The capital G at the +1 position represents the origin site of transcription.

activity. The M1/M2 of the transfections of pGL₃-control and pGL₃-basic were 1.7 and 0.05, respectively (Fig. 3). Our results indicated that the cloned 2.6-kb fragment of the 5' flanking region of the *PCAN1* gene represented promoter activity.

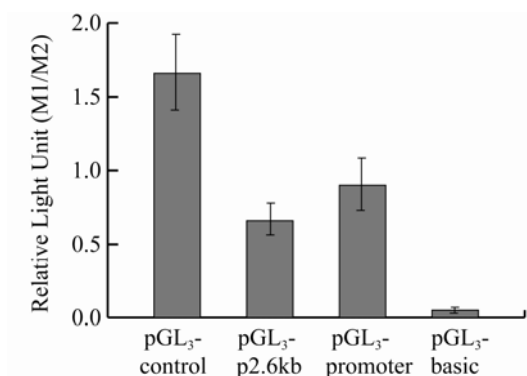


Fig. 3. The results of the promoter activity assay of pGL₃-p2.6kb using the dual-luciferase reporter assay in LNCaP cells. The 2.6-kb fragment of the 5' flanking region of the *PCAN1* gene was inserted into the pGL₃-basic vector to form pGL₃-p2.6kb, which was cotransfected with the pRL-TK plasmid (as an internal control) into LNCaP cells using lipofectimine 2000TM. The promoter activities were determined via the dual-luciferase reporter assay. The results are expressed as the relative luciferase activities (M1/M2), i.e. the ratio of firefly luciferase activity (M1) in the pGL₃ plasmid and Renilla luciferase activity (M2) in the pRL-TK plasmid. The data is the means of six individual values \pm SD. The transfections of pGL₃-control and pGL₃-basic were used as the positive and negative controls, and the pGL₃-promoter containing a SV40 promoter was used for the comparison of promoter activity.

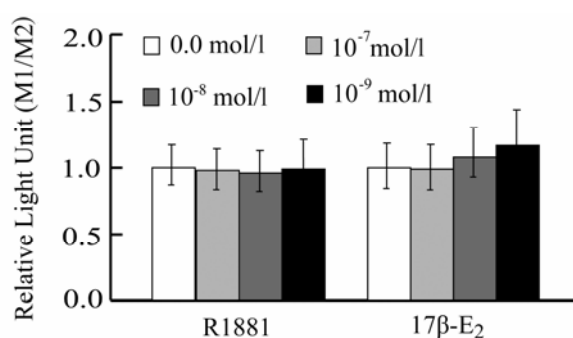


Fig. 4. The effects of all-trans-RA and 9-cis-RA on the *PCAN1* promoter activities of pGL₃-p2.6kb in LNCaP cells. pGL₃-p2.6kb was transfected into LNCaP cells treated with 10⁻⁵~10⁻⁷ mol/l all-trans-RA or 10⁻⁵~10⁻⁷ mol/l 9-cis-RA for 24 h. The effects of all-trans-RA and 9-cis-RA on the promoter activities were determined via the dual-luciferase reporter assay. The results were expressed as relative luciferase activities (M1/M2). The data is the means of six individual values \pm SD.

The effects of all-trans-RA and 9-cis-RA on *PCANI* promoter activities in LNCaP cells

24 h after the transfection of pGL₃-p2.6kb, LNCaP cells were treated with all-trans-RA (10^{-5} ~ 10^{-7} mol/l) or 9-cis-RA (10^{-5} ~ 10^{-7} mol/l) for 24 h, and then harvested for the dual-luciferase report assay. The results showed that 9-cis-RA enhanced the *PCANI* promoter activity in a dose-dependent manner, while all-trans-RA had no significant effect on *PCANI* promoter activity (Fig. 4).

The effects of R1881 and 17 β -E₂ on *PCANI* promoter activities in LNCaP cells

LNCaP cells were cultured in RPMI 1640 media containing 2% charcoal-treated FBS and cotransfected with pGL₃-p2.6kb and pRL-TK. 24 h after transfection, they were treated with R1881 (10^{-7} ~ 10^{-9} mol/l) or 17 β -E₂ (10^{-7} ~ 10^{-9} mol/l) for 24 h. The results of the dual-luciferase report assay showed that treatment with R1881 and 17 β -E₂ at different concentrations had no significant effect on *PCANI* promoter activities (Fig. 5).

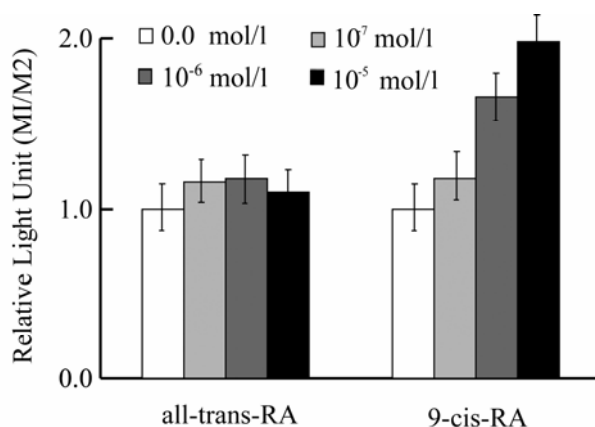


Fig. 5. The effects of R1881 and 17 β -E₂ on *PCANI* promoter activities in LNCaP cells. LNCaP cells were transfected with pGL₃-p2.6kb and treated with 10^{-7} ~ 10^{-9} mol/l R1881 or 10^{-7} ~ 10^{-9} mol/l 17 β -E₂ for 24 h. The cells were harvested for the dual-luciferase reporter assay to detect the effects of R1881 and 17 β -E₂ on the *PCANI* promoter activities. The results were expressed as relative luciferase activities (MI/M2). The data is the means of six individual values \pm SD.

Transactivation of the *PCANI* promoter by cotransfection of the expression plasmids of *NKX3.1*, *p53*, *Sp1* and *Pten*

LNCaP cells were harvested 48 h after cotransfection with pGL₃-p2.6kb and the eukaryotic expression plasmids of *NKX3.1*, *p53*, *Sp1*, *PPAR*, *Pten* or *cEBPa*. The control cells were cotransfected with pGL₃-p2.6kb and pcDNA3.1(+) plasmid. All the cells were analyzed for dual-luciferase reporter gene expression. The results in Fig. 6 show that *NKX3.1*, *p53*, *Sp1* and *Pten* had positive regulation on *PCANI* promoter activities, with the respective *PCANI* promoter

activities enhanced 1.66-, 2.48-, 2.00- and 1.72-fold compared with the *PCANI* promoter activity in the control. However, transfection with *PPAR* γ and *cEBP* α expression plasmids had no significant effect on *PCANI* promoter activities.

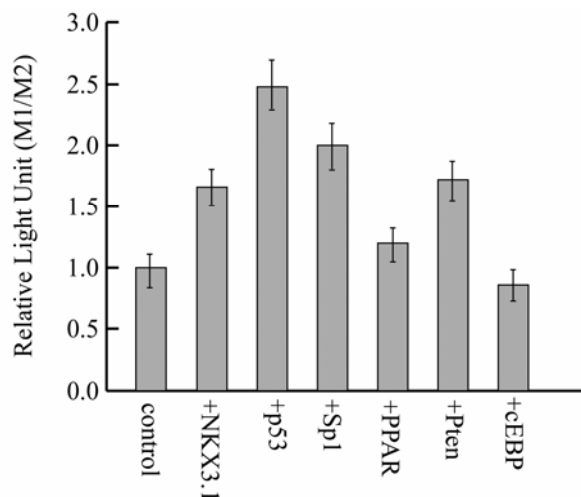


Fig. 6. The effects of *NKX3.1*, *p53*, *Sp1*, *PPAR*, *Pten* and *cEBP* α on *PCANI* promoter activities in LNCaP cells. LNCaP cells were cotransfected with pGL₃-p2.6kb and eukaryotic expression plasmids of *NKX3.1*, *p53*, *Sp1*, *PPAR* γ , *Pten* or *cEBP* α . The control cells were cotransfected with pGL₃-p2.6kb and pcDNA3.1 (+) plasmid. All the cells were harvested for the dual-luciferase reporter assay after 48 h of transfection. The results were expressed as relative luciferase activities (M1/M2). The data is the means of four individual values \pm SD.

DISCUSSION

The *PCANI* gene is highly expressed in prostate epithelial tissue. It was initially identified in a screen for prostate-specific genes, the function of which may be important in prostate cancer initiation or progression [7]. This gene has been shown to be frequently mutated or deleted in prostate tumor samples [5] and is aberrantly regulated in tumor versus normal prostate tissue, indicating that *PCANI* has a tumor suppressor role in prostate cancer [6]. However, little is known about the regulatory mechanisms of *PCANI* gene expression or the relevant regulatory elements and factors. Our research on the *PCANI* gene was started to clone the promoter and to determine the factors which could affect its activity.

In this study, 2.6 kb of the 5' flanking region of the *PCANI* gene was amplified by PCR using human genomic DNA as the template. To evaluate its promoter activity, the 2.6-kb fragment was cloned into the pGL₃-basic vector, which contains a firefly luciferase reporter gene. Our results show that pGL₃-p2.6kb provided a higher level of luciferase transcription in the LNCaP cell line than the promoter-less pGL₃-basic vector, and also had a weaker transcription level

than the pGL₃-promoter, which contained a putative strong promoter of SV40. This indicated that the cloned 2.6-kb of the 5' flanking region of the *PCANI* gene represented promoter activity. Analyses of the 2.6-kb sequence using MatInspector 2.2 on the Transfact Web site (<http://www.gene-regulation.com>) revealed potential binding sites for some important transcriptional factors (NKX3.1, P53, SP1, cEBP and PPAR/RXR heterodimers) implicated in prostate cancer. Furthermore, the analysis indicated the lack of a TATA-box within the presumed promoter region. However, we did not examine the role of downstream promoter elements in this study.

Androgens are thought to be critical regulators of prostate differentiation and function, and of prostate cancer growth and survival [8]. They are involved in prostate cancer carcinogenesis and aggressiveness via the regulation of the expression of many genes [9]. To identify whether or not the *PCANI* gene is regulated by an androgen or estrogen in prostate cancer cells, we tested the effect of an androgen (R1881) and estrogen (17 β -E₂) on 2.6-kb promoter activity. Our results showed that R1881 and 17 β -E₂ had no significant effect on *PCANI* promoter activity. The TRANSFAC software program showed that there are three progesterone response elements (GRE) within this 2.6-kb region of the *PCANI* gene, but no androgen response element (ARE) or estrogen response element (ERE) was found. Despite the presence of GREs, androgenic stimuli do not modulate the *PCANI* gene expression.

Two nuclear receptor RXR binding sites were found within the 2.6-kb region, but no RAR. To examine the effect of retinoic acid on *PCANI* promoter activity, all-trans-RA and 9-cis-RA were used to treat the pGL₃-p2.6kb-transfected LNCaP cells that express RAR and RXR [10]. Our results show that 9-cis-RA enhanced the *PCANI* promoter activity in a dose-dependent manner. 9-cis-RA is thought to be a differentiating agent and an inhibitor of carcinogenesis [11-14]. It has demonstrated anti-proliferative and/or differentiating activity in *in vitro* models of prostate cancer [15].

Analyses of the 2.6-kb sequence using MatInspector 2.2 yielded more than 500 transcription factor binding sites (Matrix similarity > 0.75). We chose some transcription factors closely related to prostate cancer to test their effect on *PCANI* promoter activities. In our experiments, the eukaryotic expression plasmids of *NKX3.1*, *p53*, *Sp1*, *Pten*, *PPAR γ* or *cEBP α* were cotransfected with pGL₃-p2.6kb into LNCaP cells to test their regulatory effect on *PCANI* promoter activities. The results showed that *PCANI* promoter activities were up-regulated by the expression of *NKX3.1*, *p53* and *Pten*, which are thought to be important cancer suppressive genes in prostate carcinogenesis. *NKX3.1* is a prostate-specific tumor suppressor. Loss of *NKX3.1* expression correlates with the initiation of prostate carcinogenesis [16] and prostate tumor progression [17]. *p53* and *Pten* are broad-spectrum tumor suppressors playing very important roles in various cancers including prostate cancer [18, 19]. Our findings that the *PCANI* gene, a highly expressed tumor suppressor, is regulated by *p53* and *Pten* raised the possibility that the apparent tissue selectivity of broad-spectrum tumor

suppressors may be generated through their regulation of tissue-specific genes to affect cell proliferation or differentiation as well as tumor progression.

In summary, we cloned a 2.6-kb fragment of the 5' flanking region of the *PCAN1* gene which represented promoter activity and was regulated by *NKX3.1*, *p53*, *Sp1*, *Pten* and 9-cis-RA in the luciferase reporter assay. Further research should be done to identify the functional cis-elements within the *PCAN1* promoter and to study the specific regulatory mechanisms.

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