

Research Paper

LncRNA *PCAT1* and its genetic variant rs1902432 are associated with prostate cancer risk

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Abstract

Emerging evidence has showed that lncRNAs and trait-associated loci in lncRNAs play a crucial role in the progression of cancer including prostate cancer (PCa). This study aimed to investigate the molecular mechanisms of lncRNA *PCAT1* involved in PCa development and its genetic variant associated with PCa risk. We applied cell proliferation and apoptosis assays to assess the effect of *PCAT1* on PCa cell phenotypes. In addition, the genome-wide profiling of gene expression was assessed from three pairs of DU145 cells transfected with *PCAT1* overexpression vector or negative control (NC) vector. Furthermore, a case-control study was conducted to explore the associations of four tagging single nucleotide polymorphisms (tagSNPs) and PCa risk in 850 PCa cases and 860 cancer-free controls. Our results showed that lncRNA *PCAT1* promoted cell proliferation and inhibited cell apoptosis. Ingenuity pathway analysis (IPA) indicated that dysregulated mRNAs induced by overexpression of *PCAT1* were primarily enriched in androgen-independent prostate tumor term and implicated in the disease and functions networks, such as cell death and survival, cell proliferation and gene expression. Besides, rs1902432 in *PCAT1* was significantly associated with increased risk of PCa (Additive model: OR = 1.19, $P = 0.014$; Co-dominant model: CC vs. TT, OR = 1.45, $P = 0.012$; Recessive model: CC vs. TT/CT, OR = 1.34, $P = 0.027$). This study suggests that *PCAT1* may act as an oncogene through promoting cell proliferation and suppressing cell apoptosis in PCa development, and genetic variant in *PCAT1* contributes to the susceptibility to PCa.

Key words: *PCAT1*; lncRNA; genetic variant; prostate cancer; molecular epidemiology

Introduction

Previous genome-wide association studies (GWASs) have identified more than 100 loci related to prostate cancer (PCa) susceptibility [1]. However, more than 90% of these loci are located in intronic or intergenic regions, whereas others are located in encoding areas [2-4]. In addition, studies have mainly focused on protein-coding genes, but definitive

evidence revealed functions of noncoding genes.

Long non-coding RNAs (lncRNAs) are defined as RNAs longer than 200 nucleotides that do not have potential to encode functional proteins. lncRNAs are frequently tissue-specific and have lower expression levels than mRNA [5]. Accumulating evidence has shown that lncRNAs play an important role in the

development of diseases. LncRNAs were involved in a variety of cellular and pathological processes such as *cis* regulation of gene expression, post-transcriptional regulation of mRNA and participation in differentiation of embryonic stem cells[6-8].

Some studies have reported that multiple lncRNAs are often altered in human urologic cancers, including PCa. Chakravarty *et al.* reported that lncRNA *NEAT1* significantly overexpressed in PCa, and promoted oncogenic growth by regulating the epigenetic signatures of target gene promoters[9]. It is well known that genetic variants located in 8q24 gene desert play an important role in susceptibility of PCa[10]. Recently, Walsh *et al.* has found that lncRNA prostate cancer-associated transcript 1 (*PCAT1*) at 8q24 participated in various biological processes in PCa cells. *PCAT1* is originally identified as an attractive biomarkers for PCa[11, 12]. Furthermore, it has been reported that upregulation of *PCAT1* implicates in cell migration, proliferation and inhibit apoptosis in hepatocellular carcinoma[13]. In addition, dysregulation of *PCAT1* has also been reported to be involved in progression of non-small cell lung cancer and bladder cancer[14, 15].

Recent studies have revealed that many GWAS or trait-associated loci are enriched in regulatory regions, including lncRNAs[16-19]. It has been reported that Single Nucleotide Polymorphism (SNP) rs3787016 in an lncRNA region at 19q13 is related with the risk of PCa[20]. Another study found that trait-associated SNP rs6983267 in 8q24 is significantly associated with the expression of lncRNA *CARLos-5*. Besides, *CARLos-5* has a long-range interaction with *MYC* enhance thus involved in the development of cancer[21]. Investigation of lncRNAs and the corresponding trait-associated SNPs may provide new insights to understand the molecular mechanisms underlying cancer susceptibility and provide valuable biomarkers[22]. In the present study, we found that lncRNA *PCAT1* promoted PCa cell proliferation and inhibited cell apoptosis. Ingenuity Pathway Analysis (IPA) revealed that the differentially expressed mRNAs induced by overexpression of *PCAT1* were primarily enriched in androgen-independent prostate tumor term and remarkably involved in the top five networks targeting cell proliferation, gene expression and so on. Additionally, tagSNP rs1902432 in *PCAT1* is a new risk locus for PCa in a Chinese population.

Methods

Study populations

All subjects were unrelated Han Chinese population. A total of 850 PCa cases and 860

cancer-free controls were recruited from the First Affiliated Hospital of Nanjing Medical University. The cases were diagnosed and histologically confirmed as PCa by at least two pathologists. All cases are not suffering from other tumors and have not taken chemotherapy or radiotherapy. The cancer-free controls were randomly collected from the local hospital in the same geographical region, and matched on sex and age (± 5 years) distribution of the PCa cases. All subjects were collected between January 2010 and June 2015 and informed consent at recruitment. A total of 10 ml EDTA blood was obtained from each subject. This study was approved by the institutional review board of Nanjing Medical University.

Construction of reporter plasmids, transient transfections

To detect the effect of *PCAT1* stable overexpression on proliferation and apoptosis of PCa cells, the full length of complementary DNA encoding *PCAT1* was synthesized and cloned into pEGFP-C1 (GENEray) using the *Bgl*III and *Kpn*I restriction sites. The constructs were confirmed by DNA sequencing analysis (Supplementary Fig.1a). The PCa cell, DU145 was transfected with *PCAT1* overexpression plasmid using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol (Supplementary Fig.1b).

Analysis of cell proliferation and apoptosis

Cells were seeded in 96-well plates after transfection with *PCAT1* overexpression plasmid. The proliferation of PCa cells was measured at certain time interval (after 12 h, 24 h, 48 h and 72h of transfection) using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The cell proliferation was represented by the absorbance value of each well at 450nm using the Infinite M200 spectrophotometer (Tecan, Switzerland). Each experiment with six replicates was repeated three times. For cell apoptosis analysis, PCa cells was collected and stained with the Annexin V-FITC Apoptosis Detection kit (Invitrogen), and the percentage of apoptotic cells was evaluated by flow cytometry.

Arraystar Human Gene Expression Array

Total RNA was extracted from DU145 PCa cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The mRNA of three pairs of DU145 cells transfected with *PCAT1* overexpression vector or corresponding NC vector was profiled with Arraystar Human Gene Expression Array. The mRNA with a *P* value ≤ 0.05 and Fold-change ≥ 2.0 is regarded as differentially expressed mRNA.

Inguinity Pathway Analysis (IPA)

The differentially expressed mRNAs in two experimental groups were uploaded in the Inguinity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA, USA).

IPA was applied to map the molecular pathways and networks based on the library of canonical pathways[23]. The canonical pathway analysis and related diseases and biofunctions analysis were performed to find out a specific pathway or function to be predominant associated with the differentially expressed genes. One sided Fisher exact test was used to assess the significance of altered pathways[24, 25].

SNP selection and genotyping

The location of *PCAT1* and its flanking 2000-bp region (3'-UTR and 5'-UTR) was pinpointed to chromosome 8, position 128,092,581 - 128,100,441. Applying the genotyping data of Han Chinese from HapMap database, seven SNPs with an allele frequency of more than 0.05 were obtained. We applied a software, Haploview 3.32 to compute linkage disequilibrium and population haplotype patterns from genotype data[26]. Using Tagger pairwise method and a tagging threshold of $r^2 > 0.8$, and four tagSNPs were selected (rs4871771, rs1902432, rs16901904 and rs710886). TaqMan allelic discrimination was applied to detect the genotypes of the four tagSNPs. The call rates for all SNPs were more than 98%. Besides, we random selected 10% of the sample to repeat genotyping results and the concordance rate was 100%.

Statistical analysis

Chi-square (χ^2) test was applied to estimate the distribution differences of demographic characteristics between cases and controls. The genotype frequencies of controls were used to calculate Hardy-Weinberg equilibrium (HWE) by goodness-of-fit chi-square test. The CCK8 results were expressed as a mean and standard deviation and Student's *t*-test was used to evaluate the difference between groups. Odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were used to evaluate the association between *PCAT1* tagSNPs and PCa risk from unconditional logistic regression analyses. The logistic regression was under four genetic models with adjustments for age and smoking. All statistical analyses were two-sided, and $P < 0.05$ was considered statistically significant. The analyses were performed using SAS (version 9.2, SAS Institute Ins., Cary, NC, USA).

Results

PCAT1 promoted prostate cancer cell proliferation

We assessed the effect of overexpression of *PCAT1* on cell proliferation in PCa cell DU145. Compared with NC vector, the expression of *PCAT1* was predominant higher in DU145 transfected with *PCAT1* expression vector (Fig.1a), which confirmed the overexpression of *PCAT1* induced by *PCAT1* expression vector. Furthermore, cell proliferation was measured at 12 h, 24 h, 48 h and 72h after transfection using CCK8 assays. As shown in Fig.1b, overexpression of *PCAT1* in PCa cell DU145 substantially increased the rate of cell proliferation, especially at 24h or later after transfection, although the effect was not observed at 12h after transfection.

PCAT1 inhibits prostate cancer cell apoptosis

To explore the effect of overexpression of *PCAT1* on cell apoptosis, we conducted flow cytometry with the DU145 cell transfected with *PCAT1* expression vector or NC vector. The results showed that overexpression of *PCAT1* significantly inhibited DU145 cell apoptosis compared with NC vector (Fig.1c). The quantifications of cell apoptosis are shown in Fig.1d. Overexpression of *PCAT1* had a remarkable effect on the cell apoptosis, suggesting *PCAT1* may play a tumor-promoting role in the PCa cell.

Identification of differentially expressed mRNA

Arraystar Human Gene Expression Array was conducted to assess the genome-wide profiling of gene expression from PCa cell DU145 transfected with *PCAT1* overexpression vector or NC vector. As shown in Supplementary Fig.2a, the heat map diagram depicted a differential gene expression pattern between overexpression of *PCAT1* and NC vector. The volcano plot displayed the differentially expressed genes by comparison of two treatment groups. The red dot indicated significantly upregulated genes whereas, the green dot meant significant downregulated gene in response to overexpression of *PCAT1* (Supplementary Fig.2b). In addition, the Supplementary Fig.3 showed the top KEGG pathways of differentially expressed genes and the VEGF signaling pathway was the most enrichment pathway.

Functional enrichment analysis of differentially expressed mRNA

The differentially expressed mRNAs in two experimental groups were uploaded in the IPA

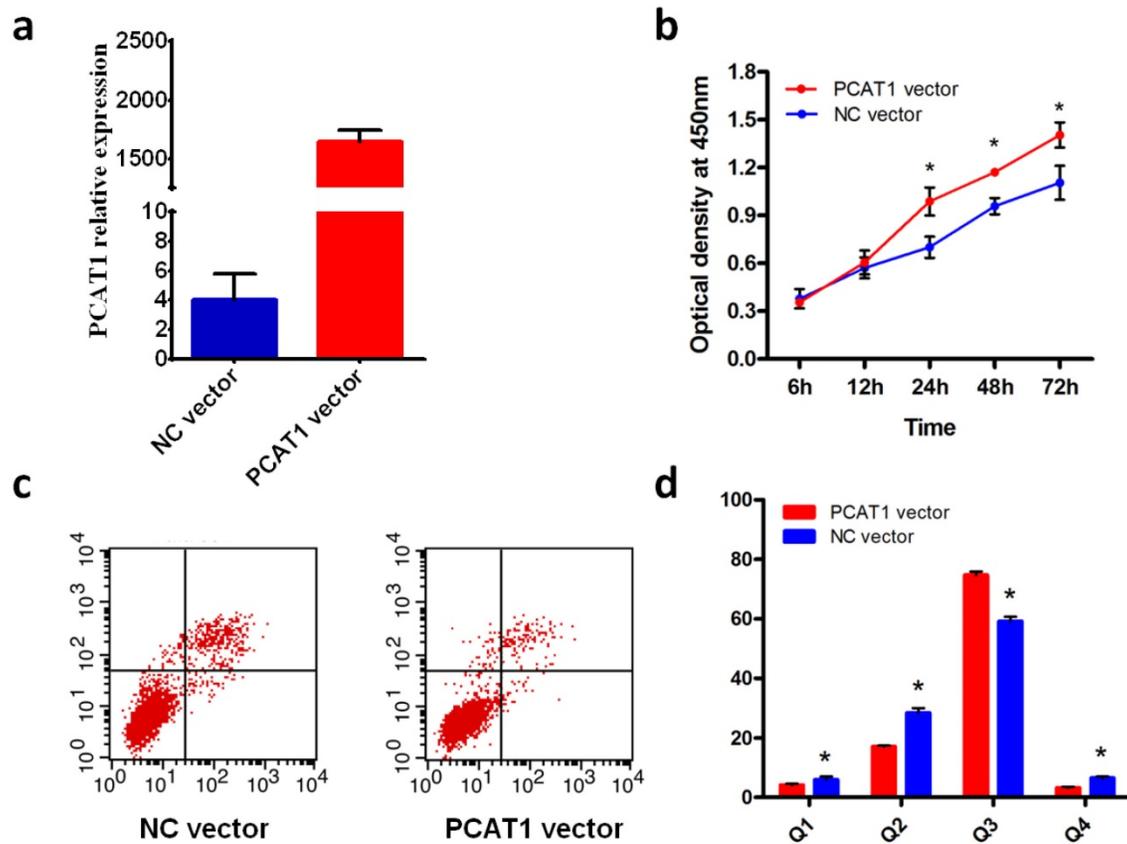


Fig.1. *PCAT1* promote DU145 PCa cell proliferation and inhibits apoptosis. **(a)** Overexpression of *PCAT1*. DU145 cells were transfected with *PCAT1* expression vector or NC vector. **(b)** *PCAT1* promote DU145 cell proliferation. Cell proliferation was measured at 12, 24, 48,72h after transfection using CCK-8 assays. Results are shown as mean ± SD of three experiments. **(c)** *PCAT1* inhibits cell apoptosis of PCa cell DU145. **(d)** Quantification of apoptosis rate of DU145 cell from c.

software to identify the implicated pathways and functional networks. As shown in **Table 1**, IPA results revealed the top five significantly enriched Disease and Biofunctions based on differentially expressed genes. Of the top five function categories, the most significant term was the androgen-independent prostate tumor ($P = 4.56 \times 10^{-5}$). Furthermore, molecular networks were applied to predict the interactions of the differentially expressed genes based on IPA molecular database. In this study, we found five enriched molecular networks, which principally related to cell death and survival, cell proliferation and gene expression (**Table 2**).

Table 1. The top five significantly enriched Disease and BioFunctions based on differentially expressed genes

| Categories | Diseases or functions annotation | P-value | Molecules |
|--|-------------------------------------|----------|-----------|
| Cancer, Organismal Injury and Abnormalities, Reproductive System Disease | Androgen-independent prostate tumor | 4.56E-05 | 5 |
| Gene Expression | Transcription | 6.04E-05 | 138 |
| Cellular Development, Hematological System Development and Function | Differentiation of blood cells | 1.03E-04 | 35 |
| Gene Expression | Expression of RNA | 1.28E-04 | 144 |
| Infectious Diseases | Infection of cells | 1.48E-04 | 81 |

Table 2. The top five networks for interactions between differentially expression genes

| ID | Top Diseases and Functions | Score | Focus Molecules |
|----|--|-------|-----------------|
| 1 | Cell Death and Survival, Hematological Disease, Immunological Disease | 128 | 119 |
| 2 | DNA Replication, Recombination, and Repair, Cell Cycle, Cellular Growth and Proliferation | 46 | 68 |
| 3 | Gene Expression, Cellular Development, Hematological System Development and Function | 44 | 66 |
| 4 | Cell Death and Survival, Cellular Growth and Proliferation, DNA Replication, Recombination, and Repair | 37 | 60 |
| 5 | Cell Death and Survival, Cellular Movement, Cellular Development | 36 | 59 |

PCAT1 rs1902432 is associated with PCa susceptibility in Chinese

Given the important role of *PCAT1* in the PCa, we investigated the associations between tagSNPs in *PCAT1* and PCa risk. The demographic and clinical characteristics of PCa patients and controls are shown in **Table 3**. Totally, 850 cases and 860 controls were included in our study. There were no significant differences in age and drinking status between case and control groups. However, more individuals with smoking and family history distributed in cases than that in controls (both $P < 0.05$), indicating that family

history and smoking play important roles in the carcinogenesis of PCa. As shown in **Supplementary Table 1**, we selected four tagSNPs (rs16901904, rs710886, rs1902432 and rs4871771), which captured all common SNPs in *PCAT1*. Of the four tagSNPs, three SNPs showed no significant deviation from HWE ($P = 0.485$ for rs710866, $P = 0.753$ for rs1902423, and $P = 0.316$ for rs4871771, respectively). However, SNP rs16901904 was deviated from HWE ($P < 0.05$). The logistic regression results of these four tagSNPs are presented in **Table 4**. In the additive model, only rs1902423 was remarkably associated with PCa risk (OR = 1.19, 95% CI = 1.04-1.37, $P = 0.014$). The other three SNPs showed no significant relationships to the risk of PCa (all $P > 0.05$). In addition, we evaluated the association between rs1902432 polymorphism and risk of PCa in other genetic models. As shown in **Table 5**, rs1902432 T > C was predominant associated with increased the risk of PCa in co-dominant model (CC vs. TT, OR = 1.45, 95%CI = 1.09 -1.94, $P = 0.012$) and in recessive model (CC vs. TT/CT, OR= 1.34, 95%CI = 1.03 -1.73, $P = 0.027$). To sum up, individual carrying rs1902432 CC genotype was significant associated with increased the risk of PCa compared with those with TT/CT genotype.

Table 3. Demographic and clinical characteristics of PCa patients and control subjects

| Variables | Cases (n = 850) | Controls (n = 860) | P |
|--------------------------|-----------------|--------------------|---------|
| Age (mean ± SD) | 71.1± 8.3 | 70.8± 7.0 | 0.411 |
| Smoking status | | | |
| No | 388 (45.9%) | 435 (50.9%) | 0.039 |
| Yes | 458 (54.1%) | 420 (49.1%) | |
| Drinking status | | | |
| Never | 615 (73.2%) | 654 (76.7%) | 0.101 |
| Ever | 225 (26.8%) | 199 (23.3%) | |
| Family history of cancer | | | |
| No | 670 (78.9%) | 769 (89.9%) | < 0.001 |
| Yes | 179 (21.1%) | 86 (10.1%) | |
| Gleason score | | | |
| <7 | 325 (38.3%) | | |
| 7 | 255 (30.1%) | | |
| >7 | 268 (31.6%) | | |
| PSA (ng/ml) | | | |
| ≤20 | 361 (42.6%) | | |
| >20 | 487 (57.4%) | | |

Table 4. Associations between four tagSNPs in *PCAT1* and PCa risk

| SNP | Cases | Controls | MAF ^a | OR(95%CI) ^b | P |
|------------|-------------------------|-------------------------|------------------|------------------------|--------------|
| rs16901904 | TT/CT/CC 564/234/41 | TT/CT/CC 563/226/71 | 0.188/0.214 | 0.88(0.75-1.03) | 0.102 |
| rs710886 | AA/AG/GG 208/421/212 | AA/AG/GG 219/439/200 | 0.498/0.489 | 1.06 (0.93-1.22) | 0.389 |
| rs1902432 | TT/CT/CC 266/423/159 | TT/CT/CC 308/414/133 | 0.437/0.398 | 1.19(1.04-1.37) | 0.014 |
| rs4871771 | TT/AT/AA 300/391/154 | TT/AT/AA 282/406/168 | 0.414/0.433 | 0.91(0.80-1.05) | 0.195 |

^a MAF (minor allele frequency) between cases and controls.

^b Logistic regression analysis with adjustment for age and smoking.

Table 5. The association between rs1902432 and the risk of PCa in four genetic models

| Genetic models | Genotypes | N(cases/controls) | Crude OR(95% CI) | Adjusted OR(95% CI) ^a | P ^a |
|----------------|-----------|-------------------|------------------|----------------------------------|----------------|
| Additive | TT | 266/308 | 1.18 (1.03-1.35) | 1.19 (1.04-1.37) | 0.014 |
| | CT | 423/414 | | | |
| | CC | 159/133 | | | |
| Co-dominant | TT | 266/308 | 1.00 | 1.00 | |
| | CT | 423/414 | 1.18(0.96-1.46) | 1.15(0.92-1.43) | 0.227 |
| | CC | 159/133 | 1.38(1.04-1.84) | 1.45(1.09-1.94) | 0.012 |
| Dominant | TT | 266/308 | 1.00 | 1.00 | |
| | CT/CC | 582/547 | 1.23(1.01-1.51) | 1.22(0.99-1.50) | 0.062 |
| Recessive | TT/CT | 689/722 | 1.00 | 1.00 | |
| | CC | 159/133 | 1.25(0.97-1.61) | 1.34(1.03-1.73) | 0.027 |

^a Logistic regression analysis with adjustment for age and smoking

Discussion

In recent years, GWASs for PCa have identified multiple risk loci, however little is known of if the SNPs themselves increased disease risk and the molecular mechanism involved in PCa[2]. The majority of GWAS or trait-associated loci are located in non-coding regions, especially 8q24 gene desert region[27, 28]. There are a variety of susceptibility loci upstream of the oncogene *MYC* in the 8q24 region[29, 30]. To date, the molecular functions of the variations in this region have not fully explored. One recent study indicated that lncRNAs originated from 8q24 locus play important roles in *MYC* regulation[31].

Another study revealed that rs7463708, a SNP located in the 8q24 region, modulated activity of the enhance of lncRNA *PCAT1*, which interacts with androgen receptor (AR) and lysine-specific demethylase 1 (LSD1) to promote PCa cell growth[19]. Emerging evidence has highlighted the need for further studies of the molecular mechanism of lncRNAs and its genetic variations underlying the development of cancers.

In this study, we have explored the effect of lncRNA *PCAT1* on PCa cell phenotypes and the associations between tagSNPs in *PCAT1* and the risk of PCa. The results showed that *PCAT1* can promote PCa cell proliferation and inhibit cell apoptosis. Overexpression of *PCAT1* contributed to multiple differentially expressed mRNA, which were mainly enriched in androgen-independent prostate tumor term and involved in the disease and functions networks, such as cell death and survival, cell proliferation and gene expression. In addition, rs1902432 is significantly associated with the risk of PCa.

Prensner *et al.* firstly identified that *PCAT1* is a novel lncRNA implicated in PCa progression through transcriptome sequencing. They discovered that prostate-specific ncRNA *PCAT1* may contribute to cell proliferation in PCa[12]. In the current study, we observed that overexpression of *PCAT1* was

remarkably increased the rate of cell proliferation in PCa cell DU145. Besides, compared with NC vector, the overexpression of *PCAT1* significantly inhibited cell apoptosis. Furthermore, IPA indicated that compared with NC vector, the differential expressed genes with the treatment of overexpression of *PCAT1* were most significantly enriched in androgen-independent prostate tumor term, which strengthened that dysregulated *PCAT1* play a key role in the progression of PCa. Interestingly, One recent study reported that *PCAT1* interacts with androgen receptor (AR) and lysine-specific demethylase 1 (LSD1) to promote PCa cell growth[19]. Besides, KEGG pathway results indicated that most significant pathway was VEGF signaling pathway, which involved in proliferation and migration of vascular endothelial cells and play a key role in tumorigenesis, including tumor initiation and function of cancer stem cells[32]. In line with the KEGG pathway results, our IPA molecular networks analysis results demonstrated that the significant five networks primarily implicated in cell death, cell proliferation and gene expression. Taken together, these results support a key role for *PCAT1* in PCa, suggesting that *PCAT1* may participate in tumorigenesis through regulating cell proliferation and apoptosis.

Although the effect of *PCAT1* on cell phenotype of multiple cancers was widely investigated, the association between polymorphisms in *PCAT1* and risk of PCa have not fully explored. One recent study has reported that three variants locate within or near several PCa associated lncRNA, including *PCAT1*, *PCAT2*, *PRNCR1* were predominant associated with PCa susceptibility[33]. We selected four tagSNPs, which captured all common SNPs in *PCAT1*, and evaluated the associations of these SNPs and the risk of PCa. We identified rs1902432 T > C in lncRNA *PCAT1* is a new susceptibility locus to PCa in Han Chinese. Significant associations between rs1902432 and PCa risk were observed in additive, co-dominant and recessive models, suggesting that rs1902432 CC genotype was associated with a significantly increased incidence of PCa. To data, there have no reports about the association of *PCAT1* polymorphism rs1902432 and the risk of diseases. Bioinformatic analyses demonstrated that rs1902432 could alter the affinity motifs to transcription factor binding (GR_known8, Nkx2_5, Nkx3_4 and Pou3f2_1). These results indicated that rs1902432 might involve in transcription regulation. Taken the importance of polymorphisms in lncRNA, examination the functional role of rs1902432 in PCa in the further studies would be warranted.

There are several strengths and novelties in the present study. Firstly, the mechanisms and clinical

implication of lncRNA *PCAT1* were widely investigated in multiple cancers, but limited evidence is available of the associations between the genetic polymorphisms in *PCAT1* and cancer risk. We found that rs1902432 T > C in *PCAT1* was a novel risk locus for genetic predisposition to PCa in a case-control study. Furthermore, Gene Expression Array and IPA results systematically pinpointed the crucially altered pathways induced by overexpression of *PCAT1*, suggesting the underlying mechanism of *PCAT1* in the carcinogenesis of PCa. Meanwhile, this study has some limitations. For example, the sample size in our study is relatively small, large well-designed studies in other independent populations are needed in the further study. Besides, whether rs1902432 has an eQTL effect on *PCAT1* was also need to investigate in the future study.

In conclusion, *PCAT1* may act as an oncogene in PCa through promoting cell proliferation and suppressing cell apoptosis. Furthermore, the genetic variation rs1902432 was significantly implicated in susceptibility to PCa, which should be noteworthy for further functional analysis in the carcinogenesis of PCa.

Supplementary Material

Supplementary figures and tables.

<http://www.jcancer.org/v09p1414s1.pdf>

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Competing Interests

The authors have declared that no competing interest exists.

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