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HNF1b is involved in prostate cancer risk via modulating androgenic hormone effects and coordination with other genes

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ABSTRACT. Prostate cancer is one of the most commonly diagnosed male malignancies. Genome wide association studies have revealed *HNF1b* to be a major risk gene for prostate cancer susceptibility. We examined the mechanisms of involvement of *HNF1b* in prostate cancer development. We integrated data from Gene Expression Omnibus prostate cancer genes from the Dragon Database of Genes Implicated in Prostate Cancer, and used meta-analysis data to generate a panel of *HNF1b*-associated prostate cancer risk genes. An RT-PCR was used to assess expression levels in DU145, PC3, LNCaP, and

RWEP-1 cells. Twelve genes (*BAG1*, *DDR1*, *ERBB4*, *ESR1*, *HSPD1*, *IGFBP2*, *IGFBP5*, *NR4A1*, *PAWR*, *PIK3CG*, *RAP2A*, and *TPD52*) were found to be associated with both *HNF1b* and prostate cancer risk. Six of them (*BAG1*, *ERBB4*, *ESR1*, *HSPD1*, *NR4A1*, and *PIK3CG*) were mapped to the KEGG pathway, and submitted to further gene expression assessment. *HNF1b*, *NR4A1*, and *HSPD1* were found to be highly expressed in the LNCaP androgenic hormone-dependent cell line. Compared to expression levels in wild-type prostate cancer cells, *NR4A1*, *HSPD1*, *ERBB4*, and *ESR1* expression levels were also found to be significantly increased in the *HNF1b*-transfected cells. We conclude that the mechanism of action of *HNF1b* in prostate cancer involves modulation of the association between androgenic hormone and prostate cancer cells. Gene-gene interaction and coordination should be taken into account to determine relationships between specific loci and diseases.

Key words: Prostate cancer; *HNF1b*; Androgenic hormone; Mechanism

INTRODUCTION

Prostate cancer is one of the most commonly diagnosed male malignancies, with the third leading morbidity rate and the sixth mortality rate in male cancer worldwide (Denmeade and Isaacs, 2004). However, the etiology of prostate cancer remains obscure. Genome-wide association (GWA) studies, characterized by high-throughput results, are useful for suggesting risk candidate genes associated with common diseases (Manolio et al., 2008). Several recent GWA studies (Gudmundsson et al., 2007; Eeles et al., 2008; Thomas et al., 2008; Takata et al., 2010; Liu et al., 2011), which are based on different ethnicities including European, Chinese, and Japanese, have universally identified hepatic nuclear factor 1 beta (*HNF1b*) as a risk locus for prostate cancer. This accumulating evidence has suggested a high-risk relationship between *HNF1b* and prostate cancer susceptibility. Despite that GWA studies have made it easy to obtain substantial information about genetic variants contributing to cancer susceptibility, they have been unable to elucidate the underlying mechanisms of the diseases or to guarantee the biological significance of the findings (Min et al., 2012).

HNF1b is located on 17q21.3 and is responsible for encoding a number of members of the homeodomain-containing superfamily of transcription factors (Tronche and Yaniv, 1992). *HNF1b* is well known to be involved in maturity-onset diabetes in the young, renal disease, and embryonic survival (Kato and Motoyama, 2009; Chen et al., 2010), but the tumorigenesis mechanism of *HNF1b* in prostate cancer is not yet clearly elucidated. In an attempt to provide a preliminary insight into this field, we hypothesized that a certain set of genes is expressed in a manner associated or coordinated with *HNF1b* in carcinogenesis. To address the hypothesis, we applied the microarray datasets from Gene Expression Omnibus (GEO) to generate genes whose expression is significantly correlated with *HNF1b*. The *HNF1b*-correlated genes were then compared with genes reported to be implicated in prostate cancer by Dragon Database (DDPC; http://apps.sanbi.ac.za/ddpc/) and our previous meta-analysis of 6 independent gene expression profiling studies about prostate cancer from GEO (Ning et al., 2011). Thus, *HNF1b*-associated prostate cancer genes were obtained. To further establish a functional mo-

lecular background of *HNF1b*, we selected genes that could be mapped to any explicit Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to evaluate the expression level by quantitative RT-PCR in 3 prostate cancer cell lines and 1 normal prostate epithelial cell line.

MATERIAL AND METHODS

GEO database

The GEO database (http://www.ncbi.nlm.nih.gov/geo/) is a public archive for high-throughput functional genomic datasets (Wilhite and Barrett, 2012). We searched GEO with the key word "*HNF1B*" and included data that met the following criteria: 1) the data were about gene expression in wild-type and induced *HNF1B* cell lines; 2) organisms were humans or rats; 3) the data provided complete microarray raw or normalized data. Finally, we found 2 datasets that met the inclusion criteria, with one for humans (GDS 1499 record) and the other one for *Rattus norvegicus* (GDS 905 record). To obtain the co-expressed genes of *HNF1b*, we downloaded text files of the included datasets by GEO accession. Using the gene ID conversion tool in Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov/), we explored the official gene names in batch for the ID references in the GEO downloaded files (Huang et al., 2009). The gene expression data of *HNF1b*-induced and -noninduced cells were compared using the Student *t*-test. The data of humans and rats were calculated separately. This file was assumed as the basis of our further comparison.

Genes implicated in DDPC

DDPC serves as a comprehensive repository that integrates data about prostate cancer from other biological research areas including molecular interactions, pathways, gene ontologies, and gene regulation at the molecular level (Maqungo et al., 2011). All the genes implicated in prostate cancer by DDPC were downloaded.

Finally, 3 groups of data, including *HNF1b* co-expression genes from GEO, prostate cancer genes from DDPC, and meta-analysis of 6 independent gene expression profiling studies about prostate cancer (Ning et al., 2011), were checked manually to identify genes that were themselves not only related to *HNF1b* but also associated with prostate cancer susceptibility.

KEGG pathways

KEGG data are widely used in pathway analysis. KEGG serves as a public archive of manually drawn pathway maps to present comprehensive and systemic interpretation for biological functions of genes (Wixon and Kell, 2000). Comprehensive searches were conducted in KEGG to select genes involved in the pathways for further analysis.

Cell lines and culture

The LNCaP cell line was kindly provided by the Institute of Molecular Biology, Nan-

Kai University, China. The DU145 cell line was purchased from the Wuhan Boster Bio-Engineering Limited Company, China. The PC3 and RWPE-1 cell lines were purchased from the XiangYa Central Experiment Laboratory, Central South University, China. DU145 and LNCap were cultured in RPMI 1640 medium (Hyclone Company, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone). PC3 were cultured in DMEM/F12 (Hyclone) supplemented with 7% FBS. RWPE-1 cells were cultured in Defined Keratinocyte-SFM (Invitrogen, USA) in the absence of FBS. All media were supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (Solarbio, China). The cells were maintained at 37°C in 5% CO_2 in a sterile incubator. An *HNF-b*-transfected PC3 cell line was also established to assess the changes in gene expression.

Plasmid construction and transfection of target cells

The plasmid vectors for *HNF1b* were purchased from GenePharma Co., Ltd. (Shanghai, China). The transfection was conducted according to the user manual. Briefly, PC3 cells were seeded on 24-well culture plates at a density of 0.5 x 10^5 cells/well 24 h prior to transfection. Cells at 80% confluence were then transfected with vectors (U6/GFP/Neo-*HNF1b*-1-homo-1759) and incubated for 48 h in serum-free medium. The green fluorescence protein was used to detect cell transfection efficiency.

Gene expression analysis

The analysis of every gene was carried out in triplicate each time, and the experiments were replicated twice with cell cultures. Glyceraldehyde-3-phosphate dehydrogenase was used to normalize target gene expression. Total RNA was extracted and reverse transcribed to cDNA following the manufacturer protocol using the RNeasy kit (Qiagen, Germany) and cDNA Synthesis kit (Fermentas, Canada), respectively. The reaction mixture contained 10 µL FastStart Universal SYBR Green Master (Roche, Germany), 6 µM forward primers, 6 µM reverse primers, and 10 ng cDNA, with RNAase-free water added to a total volume of 20 µL. The amplification and real-time analysis were run for 40 cycles with the following parameters: 95°C for 10 min for activation of FastStart Taq DNA polymerase; 60°C for 1 min for amplification and real-time analysis. The expression levels of the genes studied were determined using $2^{-\Delta\Delta CT}$.

$\Delta\Delta CT = \Delta CT^{\text{cancer/transfected cell lines}} - \Delta CT^{\text{normal cell lines}}$, and ΔCT = ΔCT(target gene) - ΔCT(endogenous reference) (Livak and Schmittgen, 2001). The primers are listed in Table 1.

Table 1. Sequences of RT-PCR primers.

Genes	Official full name	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	agaaggctggggctcatttg	aggggccatccacagtcttc
HNF1b	Hepatic nuclear factor 1 beta	cagactcacagcctgaaccc	ggtgactagttgttgaggagga
BAG1	BCL2-associated athanogene	aagaacagtccacaggaagagg	cagtgtgtcaatctcctccaag
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4	ccgaggatgagtatgtgaatga	ggttccagtagtcagggttgtc
ESR1	Estrogen receptor 1	tcctgatgattggtctcgtct	acattttccctggttcctgtc
HSPD1	Heat shock 60 KDa protein1 (chaperonin)	tgtttggagaagagggattga	gagccttgtcacctttttcctt
NR4A1	Nuclear receptor subfamily 4, group A, member 1	ctgccaatctcctcacttcc	cggagagcaggtcgtagaac
PIK3CG	Phosphoinostide-3 kinase, catalytic, gamma polypeptide	gaataggcgacagacacaatga	aaagaggaagtcaggggttagc

Statistical analysis

Data are reported as means ± SE (standard error of the mean). The analyses among the 4 cell lines were performed by ANOVA and the *post hoc* Bonferroni/Dunn test. The comparisons between the wild-type PC3 and *HNF1b*-transfected PC3 were performed by the Student *t*-test. All P values were two-sided, and $P < 0.05$ was considered to be significant. Relative expression of target genes was considered to be upregulation if the value ≥1.5, or to be undetermined if gene expression could not be determined (Das et al., 2010).

RESULTS

Genes related with *HNF1b*

A total 1019 genes showed significant differential expression in humans (GDS 1499 record) by comparing gene expression data of the wild-type and *HNF1b*-induced HEK293 cell line. A total of 1243 genes (GDS 905 record) showed significant differential expression in *R. norvegicus* by comparing the gene expression data of the wild-type and *HNF1b*-induced INS-1 cell line (Table S1).

Common prostate cancer genes in DDPC and meta-analysis

A total of 563 genes associated with prostate cancer were downloaded from DDPC. A total of 176 common genes were identified between the downloaded data and the findings of our previous meta-analysis (Table S2). Herein, we defined them as DDPC-meta prostate cancer risk genes.

Genes associated with *HNF1b* and prostate cancer

With the software available at http://jura.wi.mit.edu/bioc/tools/compare.php, we compared *HNF1b* genes and prostate cancer risk genes. No intersection was observed between the gene data of rat and 176 DDPC-meta genes, while 12 genes were found to be identical between the human genes and 176 DDPC-meta genes. They were *BAG1*, *DDR1*, *ERBB4*, *ESR1*, *HSPD1*, *IGFBP2*, *IGFBP5*, *NR4A1*, *PAWR*, *PIK3CG*, *RAP2A*, and *TPD52*. Specifically, 6 genes (*BAG1*, *ERBB4*, *ESR1*, *HSPD1*, *NR4A1*, and *PIK3CG*), which are mapped in KEGG pathway annotations, were picked out for further assessment of mRNA levels. The details of the 6 genes, including the name, GenBank accession number, and KEGG pathway description are provided in Table 2.

Expression of selected genes in the cell lines and transfected cells

The fold-changes of the genes in the 4 cell lines and the *HNF1b*-transfected PC3 cells are shown in Table 3. Although the fold-changes varied widely between PC3, DU145, and LNCaP, an upregulated expression trend of the 7 genes was observed in the prostate cancer cell lines. The increased fold-changes were particularly obvious in the hormone-dependent LNCaP cell line. Compared to the wild-type PC3, the gene expression levels mostly showed

significant upregulation after *HNF1b* transfection, particularly in the 4 genes *NR4A1*, *HSPD1*, *ERBB4*, and *ESR1* (Table 3).

Table 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of the genes associated with *HNF1b*.

Genes	GenBank accession No.	KEGG ID	KEGG pathway
BAG1	537	Hsa04141	Protein processing in endoplasmic reticulum
*ERBB*4	2066	Hsa04012	ErbB signaling pathway
		Hsa04020	Calcium signaling pathway
		Hsa04144	Endocytosis
ESR1	2099	Has04961	Endocrine and other factor-regulated calcium reabsorption
HSPD1	3329	Hsa03018	RNA degradation
		Hsa04940	Type 1 diabetes mellitus
NR4A1	3164	Hsa04010	MAPK signaling pathway
*PIK3*CG	5294	Hsa04012	ErbB signaling pathway
		Hsa04062	Chemokine signaling pathway
		Hsa04370	VEGF signaling pathway
		Hsa04510	Focal adhesion
		Hsa04620	Toll-like receptor signaling pathway

Table 3. Expression level of the genes associated with *HNF1b* in cell lines.

Genes	PC3	DU145	LNCaP	RWPE-1	*HNF1b*-transfected PC3
HNF1b	5.56 ± 0.61*	1.32 ± 0.59	18.91 ± 0.72*	0.77 ± 0.14	7.37 ± 0.98**
BAG1	3.34 ± 1.27	1.93 ± 0.36*	3.67 ± 1.66	2.23 ± 0.11	2.57 ± 0.96**
ERBB4	1.12 ± 0.26	1.89 ± 0.43	0.55 ± 0.24	0.83 ± 0.31	5.18 ± 2.07**
ESR1	4.82 ± 1.76*	ND	ND	0.83 ± 0.29	8.35 ± 2.23**
HSPD1	14.38 ± 3.2	1.07 ± 0.15	27.4 ± 2.15*	1.95 ± 0.16	39.65 ± 7.43**
NR4A1	1.83 ± 0.83	ND	8.71 ± 3.07*	2.03 ± 0.32	8.09 ± 2.39**
PIK3CG	ND	ND	ND	ND	0.18 ± 0.04**

ND = gene expression level was not determinate. **Comparisons of the wild-type PC3 and the *HNF1b*-transfected PC3 ($P < 0.05$). *Comparison of the four cell lines ($P < 0.05$).

DISCUSSION

With the integration of microarray data in GEO and the prostate cancer risk genes in DDPC, as well as the findings of our previous meta-analysis, 12 prostate cancer risk genes (*BAG1*, *DDR1*, *ERBB4*, *ESR1*, *HSPD1*, *IGFBP2*, *IGFBP5*, *NR4A1*, *PAWR*, *PIK3CG*, *RAP2A*, and *TPD52*) were identified potentially connected and related to *HNF1b*. Six of them (*BAG1*, *ERBB4*, *ESR1*, *HSPD1*, *NR4A1*, and *PIK3CG*) were found to participate in the KEGG pathways.

It was suggested that these genes may express in a manner correlated with *HNF1b*. The investigation for a panel of co-expressed genes is more likely to provide substantial information for complicated diseases than a single gene analysis. Thus, we evaluated the expression levels of *HNF1b* and the 6 genes using quantitative real-time PCR. These 7 genes were more or less found overexpressed in the prostate cancer cell lines. Meanwhile, the 6 potentially *HNF1b*-associated genes were mostly found upregulated in the *HNF1b*-transfected cells. We speculated that the main function of individual gene and genetically synergistic interactions could be the underlying molecular background of diseases. Growing evidence has demonstrated the carcinogenic character of *HNF1b* in a number of cancers, including those of the endometrium, ovary, kidney, breast, and prostate (Terasawa et al., 2006; Tommasi et al., 2009; Harries et al., 2010; Szponar et al., 2011; Setiawan et al., 2012). To the best of our

knowledge, the underlying mechanism of *HNF1b* for prostate cancer has still been poorly investigated. It is noteworthy that the fold-changes of *HNF1b* expression were remarkably higher in LNCaP, an androgen-sensitive cell line, than in the other cell lines. *HNF1b* encodes a transcription factor in humans. Song et al. (1998) reported that in rat liver, the binding of *HNF1* factor with the promoter in the dehydroepiandrosterone sulfotransferase (*Std*) gene enables transcriptional activation of Std, which could repress androgenic steroids by catalyzing sulfonation. The *Std* gene is assumed not to be tissue-specific, since it was also found to modulate androgen sensitivity in a prostate cancer cell line (Chan et al., 1998). These lines of evidence strongly suggested that *HNF1b* is likely to be involved in prostate cancer in a manner associated with steroid hormone metabolism.

In addition, the expression of *NR4A1* and *HSPD1* in prostate cancer may be closely connected with *HNF1b* as reflected by the finding that their expression fold-changes were significantly increased following transfection with *HNF1b*. *NR4A1* belongs to the steroid-thyroid hormone-retinoid receptor superfamily. It has a close association with steroid hormone metabolism, such as the regulation of the hypothalamo-pituitary-adrenal axis, and steroidogenesis (Hamid et al., 2008). Androgens can cause a rapid induction of *NR4A1* in human LNCaP prostate cancer cells (Chang et al., 1993). Furthermore, *NR4A1* plays a pivotal role in the pathway. Mitogen-activated protein kinase is a crucial signal transduction pathway associated with cancer development and controls a variety of physiological activities of cells including proliferation, differentiation, and anti-apoptosis (Johnson and Lapadat, 2002). Uemura and Chang (1998) demonstrated that *NR4A1* could reduce prostate cancer cell apoptosis and predicted its potential carcinogenesis role in prostate cancer.

HSPD1, which has long been recognized as an evolutionarily conserved stress response chaperone, has been observed to be upregulated in prostate cancer patients (Cappello et al., 2003; Skvortsov et al., 2011). The overexpression of *HSPD1* may be potentially associated with the progression and prognosis of prostate cancer (Cornford et al., 2000). *HSPD1* could suppress tumor cell apoptosis by maintaining stability of mitochondria (Ghosh et al., 2010), restraining *p53* and enhancing a common cancer gene called *survivin* (Ghosh et al., 2008; Kelly et al., 2011). *HSPD1* has also been demonstrated to be associated with early onset of hormone refractory disease in advanced prostate cancer patients under androgen ablation therapy (Castilla et al., 2010). Taken together, the possible explanation for the prostate cancer risk role of *HNF1b* could possibly be associated with modulating the relationships between androgenic hormone and prostate cancer. However, the exact mechanisms still remain to be elucidated in future studies.

The expression fold-changes of the other 4 genes (*ERBB4*, *ESR1*, *BAG1*, and *PIK3CG*) showed a coordinated association with *HNF1b*, although the changes of *BAG1* and *PIK3CG* were not as obvious as with *ERBB4* and *ESR1*. They are mapped to several important signal pathways, such as vascular endothelial growth factor signaling pathway, focal adhesion, and calcium signaling pathway. The cancer susceptibility genes with mild or weak individual effects can be aggregated into a significant risk influence (Pierce and Ahsan, 2010). Future studies are in great demand to investigate how these genes contribute to tumorigenesis as a network or through synergistic effects.

There were some limitations in this study. First, 6 genes in the absence of KEGG pathways were not examined with respect to gene expression levels, and we might have missed meaningful loci. Future studies should examine these genes. Second, our data were mainly derived from prostate cell lines, and therefore, studies are needed to extensively explore the tissue

to obtain in-depth conclusions. There were some strengths in our study. We provide a preliminary insight into the underlying mechanism of *HNF1b* prostate cancer risk by studying a gene network rather than a single gene, which may help us in interpreting a disease comprehensively.

CONCLUSION

Our study implies that the role of *HNF1b* in prostate cancer risk may be associated with androgenic hormone. Gene-to-gene interaction and coordination should be taken into account to fully explain the relationships between a locus of interest and diseases.

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Supplementary material

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