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Evidence for a role of GPRC6A in prostate cancer metastasis based on case-control and *in vitro* analyses

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Abstract. – OBJECTIVE: G protein-coupled receptor, family C, group 6, member A, (GPRC6A) is a prostate cancer (PCa) susceptibility gene and has been shown to regulate PCa progression. However, its role in PCa metastasis is largely unknown. The aim of this study was to confirm the association between GPRC6A and aggressive PCa in a case-control analysis, and to explore the function of GPRC6A in PCa metastasis in vitro.

PATIENTS AND METHODS: The association of 14 single nucleotide polymorphisms (SNPs) of GPRC6A and linked to GPRC6A were evaluated with PCa risk and aggressive PCa in 916 subjects. Metastasis behavior was determined in GPRC6A knockdown PC3 cells, and the expressions of matrix metalloproteinase (MMP)2 and MMP9 were detected. Bone transcription factor runt-related transcription factor 2 (RUNX2) and epithelial-mesenchymal transition (EMT) marker genes were examined in the GPRC6A overexpression PC3 cells.

RESULTS: Among the 14 SNPs tested in PCa patients and controls, 4 were associated with aggressive PCa (p = 0.032-0.037, odds ratio = 1.38-1.41). Both the migration and invasion abilities were reduced in PC3 cells that were transiently transfected with GPRC6A short interfering RNA (siRNA). The GPRC6A knockdown cells showed reduced activity levels of MMP2 and MMP9. Furthermore, RUNX2, EMT and ERK signaling were shown to be up-regulated in GPRC6A overexpression cells.

CONCLUSIONS: These findings suggest that GPRC6A is associated with aggressive PCa. GPRC6A knockdown inhibits the PCa cells migration and invasion, and GPRC6A overexpression promotes the EMT. It is suggested that GPRC6A may serve as a potential therapeutic target for metastatic PCa.

Key Words:
GPRC6A, Prostate cancer, Single nucleotide polymorphism, Migration, Invasion.

Introduction

G protein-coupled receptors (GPCRs) play important roles in many physiological and pathological processes, including development, hematopoiesis, angiogenesis, inflammation, mental disorders, metabolic diseases, and viral infections[1]. GPCRs and their ligands have been shown to be involved in tumor growth, invasion, and metastasis of several cancers, including prostate cancer (PCa)[2]. Accordingly, approximately 36% of all drugs target GPCRs[3]. GPCR family C, group 6, member A (*GPRC6A*) was identified and its gene cloned in 2004[4]. Since then, several reports have indicated a potential association between *GPRC6A* and PCa. Therefore, further detailed evaluation of this relationship should provide a theoretical basis for new cancer intervention based on targeting GPCRs or the GPCRs-mediated signaling pathway.

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In 2010, a genome-wide association study conducted in Japan first identified that a single nucleotide polymorphism (SNP) linked to *GPRC6A* (rs339331) is a PCa-susceptibility locus[5]. Soon after the publication of this report, this result was confirmed in multiple populations, including the Chinese population[6-9]. In human tissues, *GPRC6A* is widely expressed in the brain, peripheral tissues, kidney, pancreas, skeletal muscle, testis, and leukocytes, but is not expressed in the normal prostate[4]. The confirmed ligands of GPRC6A are L-arginine, osteocalcin, and calcium ion[10]. Most of the functional studies of *GPRC6A* conducted to date have focused on exploring the role of GPRC6A ligands in tissues or cells that express GPRC6A; for example, L-Arg or osteocalcin has been shown to stimulate insulin secretion in β-cells through GPRC6A activation[11,12]. A recent *in vitro* and *in vivo* analysis suggested that GPRC6A is a novel molecular target for regulating prostate growth and cancer progression[13]. However, there is no evidence that GPRC6A directly participates in PCa metastasis.

In this study, we sought to close this knowledge gap and evaluate the relevance of GPRC6A to PCa metastasis. We analyzed the association between *GPRC6A* and aggressive PCa in a case-control design, and performed a functional study *in vitro* to explore the role of GPRC6A in PCa metastasis. Given that GPRC6A could enhance the migration and invasion abilities of PCa cells, we further explored the expression levels of matrix metalloproteinase (MMP)2 and MMP9, which are known to promote cancer metastasis by digesting extracellular matrix (ECM). Furthermore, bone transcription factor runt-related transcription factor 2 (*RUNX2*) and epithelial-mesenchymal transition (EMT)-associated genes including E-cadherin (*E-cad*), zinc finger E-box-binding protein (*ZEB1* and *ZEB2*) and extracellular signal-regulated kinase (ERK) signaling were examined. These findings should help to elucidate the molecular pathogenic mechanism underlying aggressive PCa and determine new risk factors, with the ultimate aim of determining markers for early diagnosis and finding novel targets for intervention.

Patients and Methods

Study Population

We conducted a case-control study including 916 individuals (286 PCa patients and 630 geographically matched healthy controls). The detailed inclusion criteria for the subject recruitment for this study have been reported previously[14,15]. Clinical information regarding patients with aggressive PCa (T stage, serum prostate-specific antigen [PSA] level, and Gleason score) was obtained. Tumors with a PSA level >20 ng/ml, a Gleason score of 8 or higher, and/or a diagnosis at pathological stage III or higher were defined as aggressive PCa. In the present study, we performed age adjustment in the genotypic and allelic analyses because of a significant difference in ages between the PCa and control subjects. The Ethics Committee of the two participating hospitals approved this study and written informed consent was obtained from all individuals prior to the study.

Selection of SNPs for Genotyping

Fourteen tag SNPs at *GPRC6A* and *RFX6* (a gene adjacent to *GPRC6A*) spanning approximately 140 kb (from chr6 117,220 kb to chr6 117,360 kb) were chosen from a database of common gene variations in the Chinese population using HaploviewV4.1 software (Broad Institute, Cambridge, MA, USA) combined with the National Center for Biotechnology Information and Ensemble databases (Figure 1). A list of the selected tag SNPs and representative SNPs evaluated in this study is provided in Supplementary Table I. Blood genomic DNA was extracted from all subjects to determine the genotype. The SNPs (except for rs1606365) were genotyped using a small-amplicons method of high-resolution melting curves (HRM) combined with sequencing confirmation, as described in our previous study[14]. Because of subtle differences related to bond energy due to C/G variations, rs1606365 C/G was genotyped using an unlabeled probe method of HRM as described previously[16]. To validate the accuracy of genotyping, some samples (approximately 10%) were randomly selected for duplicate analysis, and 5 samples were randomly selected for sequencing (Beijing Genomics Institute [BGI], Beijing, China) to confirm the genotyping results. All of the primers and probes were designed using Oligo software (version 6.0; Molecular Biology Insights, Inc., Cascade, CO, USA) and were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). All of the primers used in the genotyping study are listed in Supplementary Table II.

Cell Lines and Cell Culture

RWPE-1 cells (normal prostate cells) were purchased from Shanghai Institutes for Biological

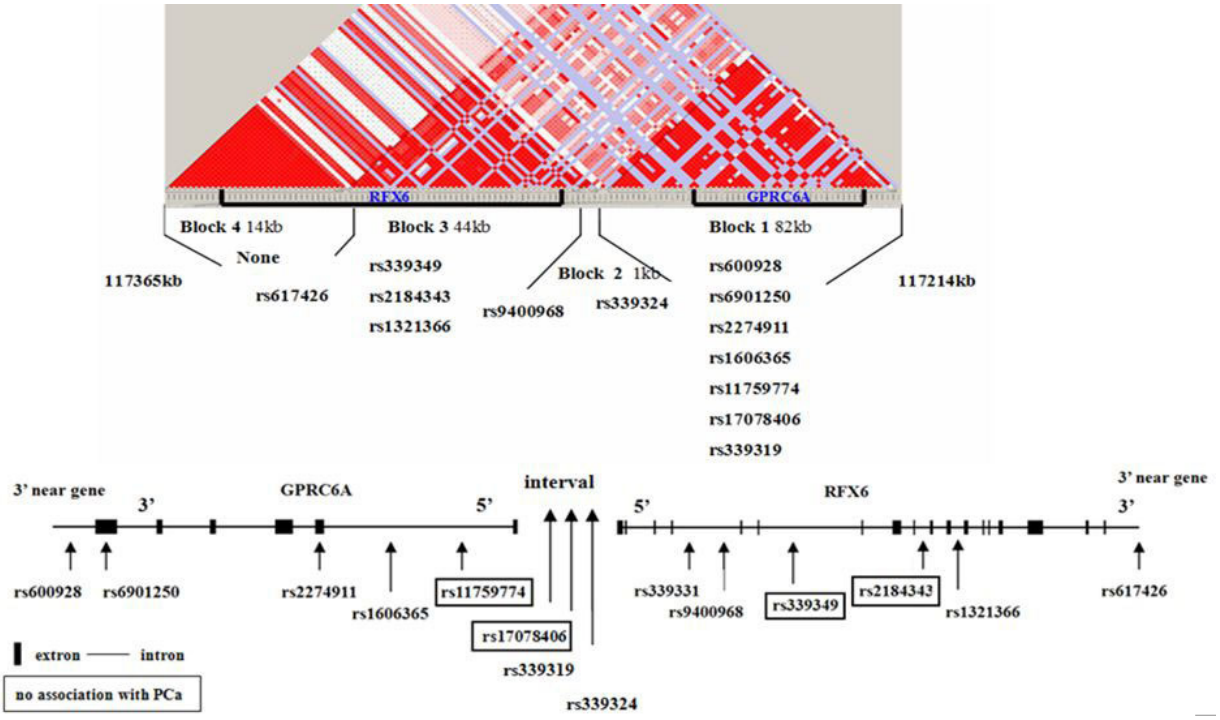

Figure 1. Location of 14 tagSNPs selected at GPRC6A and RFX6 genes. 14 tagSNPs distributed in four blocks consisting of GPRC6A/RFX6 and interval of these two genes, covering a distance of about 140kb. Among these tagSNPs, rs11759774, rs1606365, rs2274911, rs6901250, rs600928, are located at intron 1, intron 1, exon 2, exon 6 and 3 'near gene region of GPRC6A, respectively. Rs17078406, rs339319 and rs339324 are at interval of GPRC6A and RFX6. Rs339331 and rs9400968 are at intron 4, rs339349 is at intron 6, rs2184343 is at intron 9, rs1321366 is at intron 11, rs617426 is at 3 'near gene of RFX6. Except rs11759774, rs17078406, rs339349 and rs2184343, other SNPs were associated with PCa in our study.

Sciences, Chinese Academy of Sciences (Shanghai, China), PC3, LNcap and the human embryonic kidney (HEK) 293T cells were purchased from the Institute of Basic Medical Science, Chinese Academy of Medical Science and Peking Union Medical College (Beijing, China), and VCaP cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were used within 6 months after receipt or resuscitation of the frozen vial. The commercial supplier authenticated cell lines by DNA (STR) profiling and confirms they are pathogen-free, in addition to be tested by morphological characteristics and the special protein expressions (e.g. expression of androgen receptor) in our laboratory (Supplementary Figure 1). RWPE-1 cells were cultured using keratinocyte serum-free medium with insulin and growth supplementation (KGM™-CD BulletKit™, Lonza Walkersville, Inc., Basel, Switzerland). The three PCa cell lines were maintained in F12K, Dulbecco's modified Eagle medium (DMEM), and Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% carbon-dextran-filtered fetal bovine serum (charcoal-stripped FBS, C-FBS), respectively. HEK 293T cells were used to package lentivirus and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). All the cells were cultured in a humidified incubator of 5% CO_2 at 37 °C. The cell culture medium DMEM, RPMI 1640 and FBS were products from Gibco (Invitrogen, Carlsbad, CA, USA).

Gene Knockdown by Short Interfering RNA (siRNA) Transfection

Three *GPRC6A* siRNAs and one control siRNA were synthesized by Ribobio Company (Guangzhou, China). One optimal *GPRC6A* siRNA that could combine with the target sequence GAATTACAATGAAGCCAAA was selected after transient transfection with different concentrations of siRNA (Supplementary Figure 2). According to the manufacturer instructions for the use of Lipofectamine™ RNAiMAX (Invitrogen, Camarillo, CA, USA), the combination of reverse

and forward transfection methods was used to interfere with the expression of *GPRC6A* according to the instruction. The knockdown efficacy was confirmed using Western blotting. Three independent experiments were performed.

Scratch Wound-Healing Assay

PC3 cells that were transiently transfected with *GPRC6A* siRNA or universal siRNA as a control were used to determine the effect of GPRC6A on the migratory behavior of PCa cells. 48 hours after transfection, the cells were gently scratched in parallel and crosswise across the well with a 10-µl pipette tip and then washed twice with D-PBS to remove the detached cells; fresh culture medium was then added to the well. Multiple images were captured for each well at 24 hours after the scratch was made using an inverted-phase contrast microscope with a digital camera ECLIPSE Ti-S (Nikon, Tokyo, Japan). Three independent experiments were performed.

Transwell Migration and Invasion Assays

Transwell cell culture inserts (24-well inserts, 8-mm pore size; Corning Life Sciences, Corning, NY, USA) were used to analyze the migration and invasion abilities of PC3 cells with and without GPRC6A expression. For the migration assay, 5×10^5/ml of PC3 cells that were transiently transfected with *GPRC6A* siRNA or universal siRNA as a control were seeded on the top of the inserts with 200 µl DMEM/F12 medium containing 0.1% bovine serum albumin. The bottom chamber was filled with 700 µl DMEM/F12 medium containing 10% C-FBS. After incubation for 16 hours, the cells were fixed in 4% paraformaldehyde then were stained with 0.1% crystal violet, and images were captured after scraping the upper cells. The inserts were washed with PBS three times, and the migrated cells were extracted using 100 µl of 33% ethanoic acid and the absorbance was measured at 590 nm. The same procedures described above were used to assay the invasion ability of cells, except that the upper polycarbonate membranes of the filters were pre-coated with 60 µl of 85 µg/cm^2 Matrigel and the cells were incubated for 20 hours[17].

Gelatin Zymography

At 48 hours after transfection, the cells were cultured in serum-free DMEM for 24 hours and the conditioned medium was collected. The supernatants were collected and the protein content in the medium was determined using the BCA method. Equal amounts of protein were fractionated by 10% SDS-PAGE with 1 mg/ml gelatin added to the gel. Gelatin zymography was carried out with the MMP zymography assay kit (Applygen Technologies Inc., China). Gels were incubated in incubation solution after being eluted and rinsed according to the manufacturer instructions. The gels were then stained with 0.05% Coomassie Brilliant Blue solution for 3 hours and destained with a destaining solution containing methanol and ethanoic acid to visualize the bands.

Vector Constructing, Lentivirus Packaging and Stable Cell Lines Screening

The PCDH-CMV-MCS-EF1 lentivector with green fluorescent protein (GFP) and puromycin markers was used to construct *GPRC6A* overexpression vector. Full cDNA of *GPRC6A* were generated by splicing and overlapping extension the fragments using human total DNA as template. Lentivirus packaging was performed using the third generation systems (other helper plasmids were vsv-g, rsrv and pmd2.0g) after the constructed vector was sequenced to confirm its correctness. The HEK 293T cells were transfected with three helper plasmids and *GPRC6A* overexpression vector or empty PCDH control vector using Lipofectamine® LTX with PlusTM Reagent (Life Technologies, MD, USA) according to the manufacturer's instruction. The packaged lentivirus was used to infect PC3 cells and the puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium after being infected 48hours. The stable cells those overexpress *GPRC6A* were reserved after the no fluorescence-cells were killed by puromycin. The overexpression effect was confirmed by using Western blotting and real-time PCR methods (Supplementary Figure 3).

RNA Isolation, Reverse Transcription and Real-time PCR

Total RNA was extracted from the PC3 cells those overexpressed GPRC6A and control cells transfected with empty vector after lysis by using Trizol (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse transcribed to cDNA using PrimeScript™ RT Master Mix (TaKaRa Bio, Shiga, Japan). The SYBR® Premix Ex Taq™ II kit (TaKaRa Bio, Otsu, Shiga, Japan) was used to detect the expressions of *RUNX2* and EMT-associated genes according to the manufacturer's instructions. The sequences of primers in real-time are listed in Table I. The ratio of expressions of EMT-genes and internal control GAPDH was the relative expressions. The ΔΔCT method was used

Table I. Real-time PCR primers of EMT related genes.

Gene	Primer
RUNX2F	CCGAGACCAACAGAGTCATTTAAG
RUNX2R	TGGTGTCACTGTGCTGAAGAG
E-cad F	AGACCAAGTGACCACCTTAG
E-cad R	GCAGCAGAATCAGAATTAGCAAAG
ZEB1F	CATAAATCAGGAAGAGATCAAAGAC
ZEB1R	TAGGAGCCAGAATGGGAAAAG
ZEB2F	GACATAAATACGAACACACAGGAA
ZEB2R	GCTTGCCACATTTATCACACTG
GAPDH Human F	CAACAGCCTCAAGATCATCAGCA
GAPDH Human R	TGGCATGGTCTGTGGTCATGAGT

RUNX2: Runt-related transcription factor 2; E-cad: E-cadherin; ZEB: zinc finger E-box-binding protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. F: Forward primer. R: Reverse primer.

to evaluate the fold differences between different groups. Three independent experiments were performed and all samples were run in triplicate.

Western Blotting

To determine the expression levels of GPRC6A, MMP2, MMP9, ERK and p-ERK in the different cell lines, a western blotting method was used as described previously[18]. Cells were lysed with cold lysis buffer with protease inhibitors and the supernatants were collected. For the detection of ERK signaling, cells were lysed with cold lysis buffer added protease and phosphatase inhibitors. Protein concentration was assayed using a bicinchoninic acid (BCA) kit (Applygen Technologies Inc., Beijing, China). The primary antibodies used were as follows: anti-GPRC6A (Abcam, Inc., Cambridge, UK), anti-MMP2, anti-ERK, anti-pERK (Bioworld Technology, Inc. St. Louis Park, MN, USA), anti-MMP9 (PTG, Proteintech Group, Inc., Chicago, IL, USA) and anti-β-actin (Ameribiopharma, Inc., Boulder, CO, USA).

Statistical Analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were tested for each SNP separately among control subjects using Pearson's χ^2 with a cut-off *p*-value of >0.05. Allelic- and genotypic-based association analyses of the tag SNPs with PCa risk were performed using a logistic regression method to obtain the odds ratios (ORs) and 95% confidence intervals (CIs). The Statistical Package for the Social Sciences software package, version 16.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses. Haplotype-based association analysis was also performed using Haploview V 4.1 software. Power values were calculated by Pawe V1.2 software. The Student's *t*-test was used to determine *p*-values in the comparison between two groups using GraphPad Prism version 5 (GraphPad Software, Inc.). Except for the HWE test, a p-value < 0.05 was considered to be significant in all statistical tests.

Results

Association between GPRC6A and PCa

The mean ± SD age of the patients was 72.3 ± 7.5 years (range 46 to 93), and the mean ± SD age of control subjects was 70.5 ± 7.9 years (range 58 to 94), indicating a significant age difference between the two groups ($p = 0.002$). The demographic characteristics of all study subjects are listed in Supplementary Table III. Among the control subjects, the genotype frequencies of all 14 SNPs did not deviate from HWE ($p > 0.05$; Table II). Association analysis between alleles of the tag SNPs and PCa showed that 10 of the 14 tag SNPs were significantly associated with PCa risk (age-adjusted OR = 1.24-1.39, $p = 0.002$-0.045). The genotype frequencies analysis showed that 9 of the tag SNPs were associated with increased PCa risk in all three genetic models (Table III). Two haplotype blocks identified by the 14 SNPs were inferred in linkage disequilibrium (D′) evaluation (Supplementary Figure 2). Haplotype-based association analysis showed that 3 haplotypes were strongly associated with PCa (OR = 1.369, 0.655, and 1.526; $p = 0.005$, 7×10^{-4} and 7×10^{-4}, respectively). The haplotype consisting of 7 risk alleles (ATGTGGG) of the SNPs confirmed in our study showed a significant difference between the patients and control subjects (OR = 1.513; $p = 6 \times$

Table II. Association analysis between alleles of tagSNPs and PCa in northern Chinese men.

SNPs ID	Allele	Risk allele	Gene	RAF		HWE	Unajusted allelic OR		Age-adjusted allelic OR		Power
				Case	Control	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*	
rs600928	C/T	C	GPRC6A	0.582	0.512	0.364	1.34 (1.09-1.64)	**0.005**	1.33 (1.08-1.64)	**0.006**	0.795
rs6901250	G/A	A	GPRC6A	0.504	0.448	0.787	1.25 (1.02-1.54)	**0.031**	1.24 (1.01-1.52)	**0.045**	0.600
rs2274911	T/C	T	GPRC6A	0.574	0.51	0.371	1.30 (1.06-1.59)	**0.012**	1.29 (1.05-1.59)	**0.014**	0.720
rs1606365	C/G	G	GPRC6A	0.462	0.384	0.519	1.39 (1.13-1.70)	**0.002**	1.39 (1.13-1.71)	**0.002**	0.882
rs11759774	A/G	A	GPRC6A	0.957	0.948	0.809	1.22 (0.76-1.97)	0.417	1.16 (0.71-1.87)	0.554	0.131
rs17078406	T/C	T	Interval	0.688	0.652	0.891	1.18 (0.94-1.47)	0.147	1.20 (0.96-1.50)	0.117	0.327
rs339319	C/T	C	Interval	0.58	0.508	0.070	1.34 (1.10-1.64)	**0.004**	1.33 (1.09-1.64)	**0.006**	0.816
rs339324	T/C	C	Interval	0.526	0.446	0.454	1.38 (1.13-1.70)	**0.002**	1.36 (1.11-1.68)	**0.004**	0.889
rs339331	T/C	T	RFX6	0.694	0.618	0.643	1.40 (1.13-1.73)	**0.002**	1.35 (1.09-1.68)	**0.006**	0.882
rs9400968	A/G	G	RFX6	0.636	0.558	0.301	1.39 (1.12-1.71)	**0.002**	1.38 (1.11-1.70)	**0.003**	0.880
rs339349	G/A	A	RFX6	0.048	0.038	0.223	1.29 (0.79-2.09)	0.305	1.26 (0.77-2.07)	0.350	0.170
rs2184343	G/T	T	RFX6	0.051	0.048	0.597	1.05 (0.65-1.69)	0.836	0.98 (0.60-1.59)	0.937	0.059
rs1321366	G/A	G	RFX6	0.700	0.621	0.185	1.43 (1.15-1.77)	**0.001**	1.39 (1.12-1.73)	**0.003**	0.906
rs617426	G/T	G	RFX6	0.677	0.608	0.872	1.36 (1.1-1.68)	**0.004**	1.33 (1.08-1.65)	**0.008**	0.809

$p < 0.05$ are in bold. RAF, risk allele frequency.

Table III. Association analysis between the different genetic models of tag SNPs and PCa in northern Chinese men.

SNPs ID	Additive model (df=2)	Unadjusted Genotypic OR				Age-adjusyed Genotypic OR			
		Dominant model (11+12 vs. 22)		Recessive model (11 vs. 12+22)		Dominant model (11+12 vs. 22)		Recessive model (11 vs. 12+22)	
	p	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*
rs600928	**0.01**	1.28 (0.89-1.83)	0.18	1.63 (1.20-2.23)	**0.002**	1.24 (0.86-1.78)	0.251	1.67 (1.22-2.28)	**0.001**
rs6901250	0.10	1.34 (0.96-1.85)	0.083	1.36 (0.97-1.91)	0.075	1.29 (0.93-1.80)	0.131	1.36 (0.96-1.92)	0.08
rs2274911	**0.04**	1.40 (0.97-2.00)	0.071	1.45 (1.06-1.97)	**0.020**	1.34 (0.93-1.93)	0.112	1.47 (1.08-2.02)	**0.016**
rs1606365	**0.01**	1.46 (1.07-1.98)	**0.017**	1.70 (1.18-2.45)	**0.004**	1.40 (1.02-1.90)	**0.035**	1.81 (1.25-2.62)	**0.002**
rs11759774	0.55	-	-	1.19 (0.73-1.95)	0.489	-	-	1.13 (0.69-1.85)	0.639
rs17078406	0.35	1.26 (0.79-2.03)	0.334	1.22 (0.91-1.64)	0.183	1.22 (0.76-1.97)	0.415	1.27 (0.95-1.71)	0.112
rs339319	**0.01**	1.47 (1.02-2.12)	**0.041**	1.53 (1.12-2.08)	**0.008**	1.41 (0.97-2.04)	0.069	1.56 (1.14-2.13)	**0.006**
rs339324	**0.01**	1.43 (1.02-2.00)	**0.036**	1.67 (1.2-2.34)	**0.003**	1.37 (0.97-1.92)	0.072	1.69 (1.2-2.37)	**0.003**
rs339331	**0.003**	1.41 (0.9-2.21)	0.130	1.65 (1.26-2.19)	**0.001**	1.34 (0.86-2.10)	0.202	1.57 (1.2-2.10)	**0.002**
rs9400968	**0.001**	1.22 (0.83-1.8)	0.310	1.75 (1.3-2.35)	**2.4×10^{-4}**	1.20 (0.81-1.78)	0.358	1.74 (1.28-2.35)	**3.4×10^{-4}**
rs339349	0.56	-	-	2.23 (0.31-15.89)	0.424	-	-	2.81 (0.39-20.3)	0.306
rs2184343	0.98	-	-	1.17 (0.11-12.97)	0.898	-	-	0.72 (0.05-9.59)	0.804
rs1321366	**0.002**	1.35 (0.85-2.13)	0.202	1.69 (1.27-2.25)	**3.4×10^{-4}**	1.29 (0.81-2.04)	0.283	1.65 (1.23-2.20)	**0.001**
rs617426	**0.01**	1.21 (0.8-1.83)	0.366	1.62 (1.22-2.16)	**0.001**	1.16 (0.77-1.76)	0.476	1.60 (1.19-2.13)	**0.002**

1, risk allele and 2, non-risk allele for the dominant mode (11 + 12 vs. 22) and the recessive mode (11 vs. 12 + 22). -The genetic models were not analyzed due to one of the genotype frequencies was less than 0.05. $p < 0.05$ are in bold.

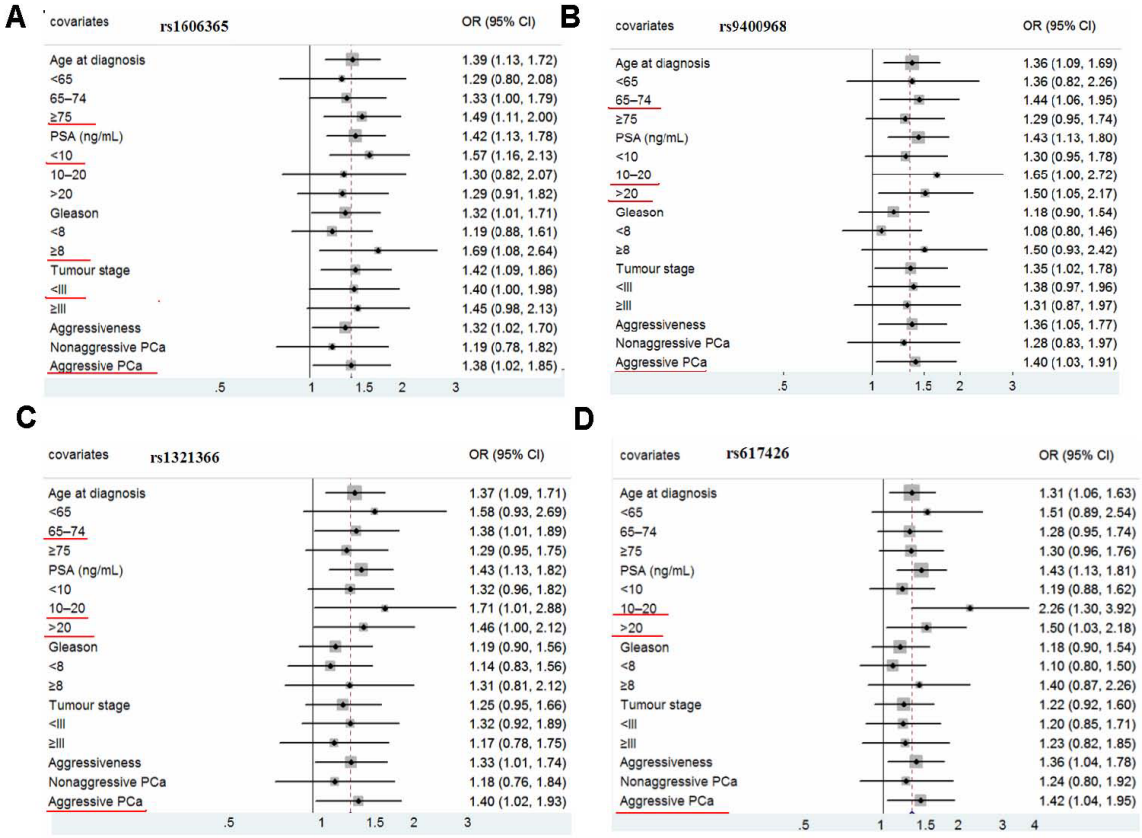

Figure 2. Odds ratios with confidence intervals of the association between 4 risk loci and age at diagnosis, Gleason score, PSA level, disease stage and disease aggressiveness. ***(A)*** Compared with controls, rs1606365 was associated with age at diagnosis more than 75 years, PSA less than 10 ng/ml, Gleason score 8 or greater, disease stage less than III and aggressive PCa (OR 1.38); ***(B)*** rs9400968 was associated with age at diagnosis 65-74 years, PSA more than 10 ng/ml and aggressive PCa (OR 1.40); ***(C)*** rs1321366 was associated with age at diagnosis 65-74 years, PSA more than 10 ng/ml and aggressive PCa (OR 1.40); ***(D)*** rs617426 was associated with PSA more than 10 ng/ml and aggressive PCa (OR 1.42).

10^{-4}) (Table IV). Association analysis of the risk tag SNPs and clinical phenotypes suggested that 4 SNPs were associated with more than two clinical indices, and all were associated with aggressive PCa (Supplementary Tables IV to VII). Figure 2 shows the ORs with CIs for the association between these 4 risk loci and clinical covariates.

Expression Levels of MMP2 and MMP9 in PCa Cells

The results of GPRC6A protein expression analysis showed that among the three PCa cell lines, the expression of GPRC6A was highest in VCaP cells, which was about 6-times higher than that in normal prostate epithelial cells (RWPE-1). The expression of GPRC6A in PC3 cells was about 3-times greater than that of normal cells. However, there was no significant difference in the GPRC6A expression level between LNcap and RWPE-1 cells. The patterns of protein expression of MMP2 and MMP9 in VCaP, PC3, and LNcap cells were roughly consistent with those of GPRC6A (Figure 3).

Effect of GPRC6A knockdown on the Migration and Invasion Abilities

The results of the scratch experiment showed that the number of PC3 cells treated with *GPRC6A* siRNA migrating to the blank scratch area was reduced compared with those treated with control siRNA (Figure 4A). The transwell migration and invasion assay results also indicated that the number of PC3 cells transfected with *GPRC6A* siRNA passing through the polycarbonate membranes was lower than that in the group of cells transfected with control siRNA. Furthermore, the absorbance value of acetic acid-solubilized crystal violet was significantly

Table IV. Haplotype block and haplotype-based association analysis of tag SNPs at GPRC6A and RFX6 genes with PCa.

Haplotypes	Case (freq)	Control (freq)	Chi-squared	p-value	OR (95% CI)
Block 1					
A T C A T	0.061	0.059	0.05	0.824	1.053 (0.671-1.651)
A T G A T	0.446	0.379	7.925	**0.005**	1.369 (1.100-1.705)
G T C G T	0.037	0.057	2.673	0.102	0.640 (0.373-1.097)
G C C A C	0.301	0.349	2.613	0.106	0.826 (0.655-1.042)
G C C A T	0.122	0.141	0.795	0.373	0.864 (0.626-1.192)
Block 2					
C A G G A	0.249	0.357	11.458	**7×10^{-4}**	0.655 (0.513-0.838)
T G G G G	0.589	0.555	11.458	**7×10^{-4}**	1.526 (1.194-1.950)
7 risk loci					
A T G T G G G	0.404	0.340	11.672	**6×10^{-4}**	1.513 (1.192-1.920)
G T C T G G G	0.037	0.055	1.813	0.178	0.692 (0.404-1.186)
G C C C A A T	0.231	0.322	10.392	**0.001**	0.657 (0.509-0.849)
G C C T G G G	0.123	0.121	0.227	0.634	1.084 (0.779-1.508)

Risk loci are rs6901250, rs2274911, rs1606365, rs339331, rs9400968, rs1321366 and rs617426 that were associated with PCa in our study. Block 1 contained the risk alleles of rs6901250, rs2274911, rs1606365, rs11759774 and rs17078406. Block 2 contained the risk alleles of rs339331, rs9400968, rs339349, rs2184343 and rs1321366. 7 risk loci composed by the risk alleles of rs6901250, rs2274911, rs1606365, rs339331, rs9400968, rs1321366 and rs617426. $p < 0.05$ are in bold.

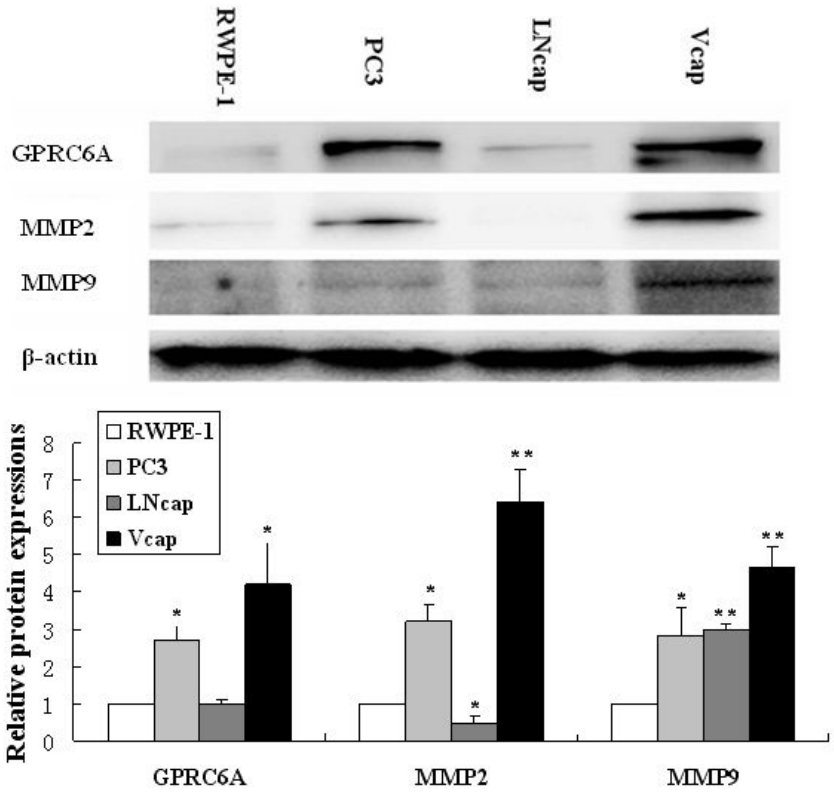

Figure 3. Expressions of GPRC6A, MMP2 and MMP9 in normal prostate cell and PCa cell lines. Whole cell lysates were analyzed by immunoblotting with anti-GPRC6A, MMP2 and MMP9 antibodies. The expression of GPRC6A and MMP2 in bone-derived cells PC3 and Vcap was higher than that in normal prostate cells RWPE-1 and supraclavicular lymph node-derived cells LNcap. The expression of MMP9 in PCa cells was higher than that in normal prostate cells RWPE-1. *$p < 0.05$, versus control prostate cell RWPE-1. **$p < 0$ .01, *versus* control prostate cells RWPE-1.

different between the two cell lines ($p < 0.05$; Figures 4B and 5A,B). These results suggested that GPRC6A promotes the migration and invasion abilities of PCa cells.

Effect of GPRC6A Knockdown on the exPression and Activity of MMP2/MMP9

The expression levels of MMP2 and MMP9 detected in *GPRC6A* siRNA-treated PC3 cells

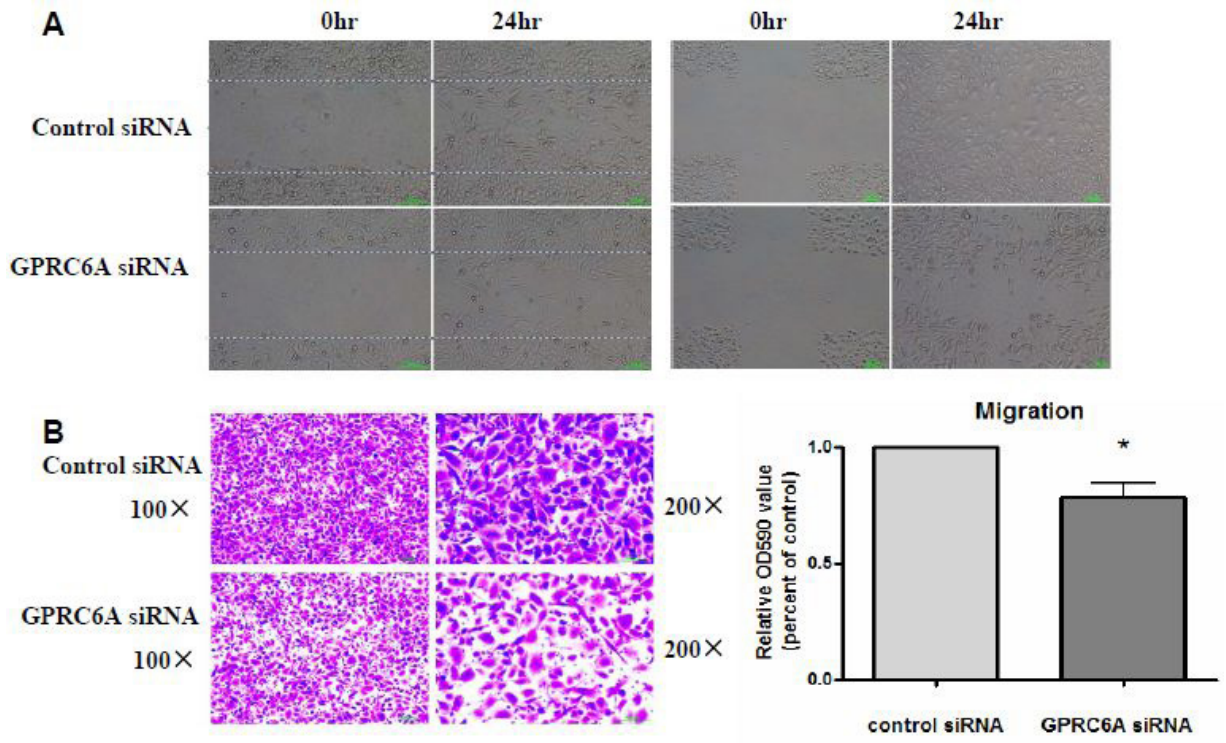

Figure 4. Suppression of migration in PCa cells transfected GPRC6A siRNA. After transfection of PC3 cells with GPRC6A siRNA and control siRNA for 48 hours, scratch wound healing assay ***(A)*** and transwell migration detection ***(B)*** was performed to determine the migration ability of PCa cells. The value of absorbance at OD_{590} showed the number of migrated cells in GPRC6A siRNA group was less than that in control siRNA group. $^{*}p < 0.05$, *versus* control siRNA group.

were lower than those in the control siRNA-treated PC3 cells, in accordance with the difference in the expression of GPRC6A in the two cell groups (Figure 5C). Gelatin zymography analysis showed that the activity of MMP2 and MMP9 in the PC3 control siRNA cell culture medium was significantly higher than that in the PC3 *GPRC6A* siRNA cell culture medium ($p < 0.05$; Figure 5D).

Expressions of pERK and EMT-Genes in GPRC6A Overexpression Cells

P-ERK was significantly enhanced in the *GPRC6A* overexpression PC3 cells ($p < 0.01$; Figure 6A). Expressions of *RUNX2*, *ZEB1* and *ZEB2* in *GPRC6A* overexpression cells (PC3-GPRC6A) were higher than those in the control cells (PC3-PCDH). However, expression of *E-cad* was reverse ($p < 0.05$; Figure 6B-E).

Discussion

As one of the cancer hallmarks, genome instability or mutation is the primary genetic mechanism that can promote progression, including activating the invasion or metastasis of cancer cells[19]. A systematic analysis of somatic mutations in cancer genomes revealed that nearly 20% of all human tumors harbor mutations in the genes encoding GPCRs[20]. In this study, we systematically screened SNPs of *GPRC6A* in patients and healthy subjects, and explored the role of GPRC6A in PCa metastasis *in vitro*. Our data showed that several SNPs in or adjacent and linked to *GPRC6A* were associated with aggressive PCa. Furthermore, knockdown of GPRC6A expression could reduce the abilities of migration and invasion in the PC3 cells, and overexpression of GPRC6A could enhance the EMT and ERK signaling.

Previously[9], we demonstrated an association between the SNP rs339331 of *RFX6* with PCa. In order to find the linked regions at the *GPRC6A* locus, identify rare variants, and further explore the role of functional variants of *GPRC6A* in PCa development, we systematically analyzed the associations of 14 SNPs of *GPRC6A* or *RFX6* with PCa risk and clinical covariates in the northern Chinese population. Ten of the 14 tag SNPs and

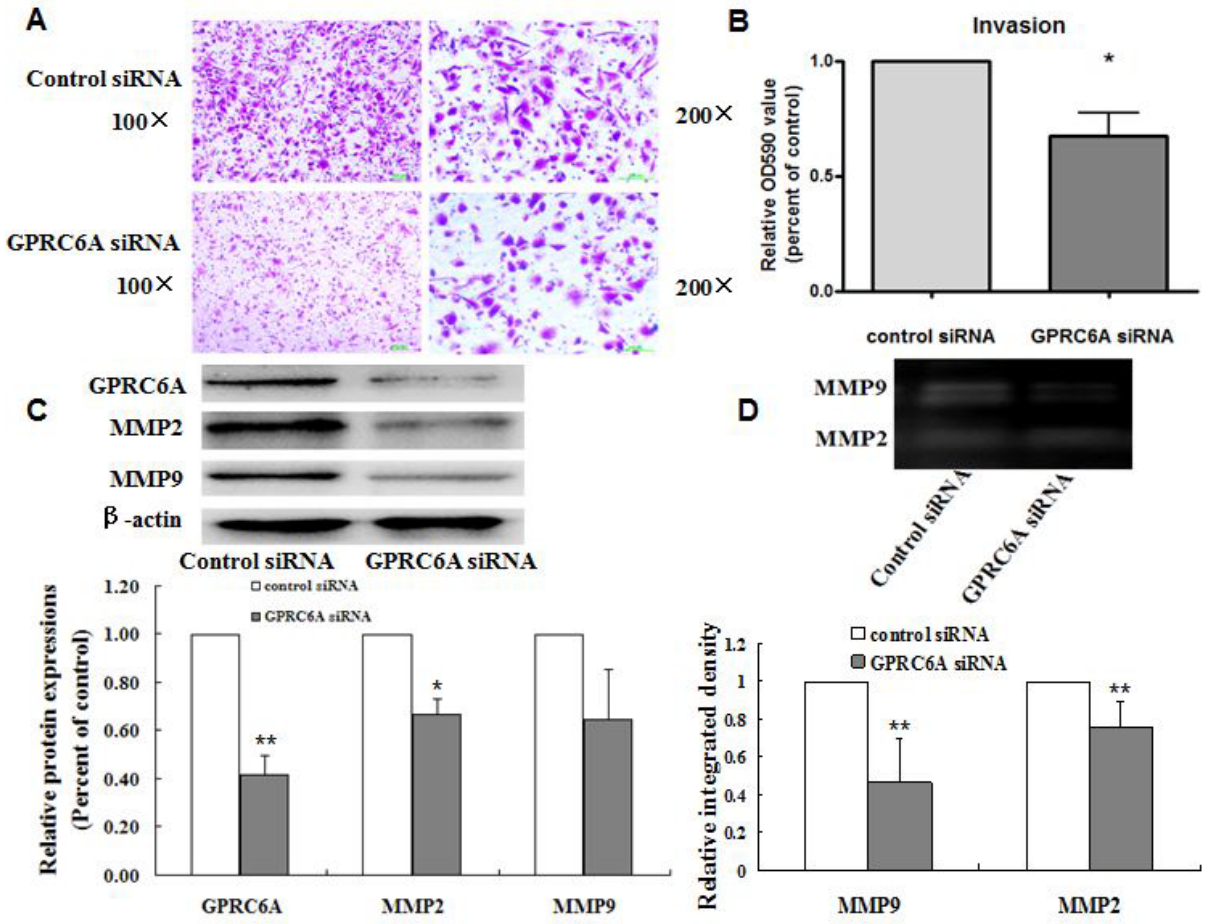

Figure 5. Suppression of invasion and expression of MMP2/MMP9 in PCa cells transfected GPRC6A siRNA. The invasion ability was estimated using transwell coated with matrigel (A) and the cells cut through the inserts membrane were extracted by using 33% ethanoic acid and measured for absorbance at OD_{590} (B) after transfection of PC3 cells with GPRC6A siRNA and control siRNA for 48 hours. Simultaneously, the expressions of MMP2/MMP9 in PC3 cells (C) and the activity of MMP2/MMP9 in cell culture medium (D) were determined after transfection of PC3 cells with GPRC6A siRNA and control siRNA for 48 hours. *$p < 0.05$, *versus* control siRNA group. **$p < 0.01$, *versus* control siRNA group.

three haplotypes that covered the full *GPRC6A/RFX6* gene region were associated with PCa risk. Although we failed to identify the specific location of the PCa association, further analysis of risk tag SNPs and clinical-related phenotypes suggested that 4 SNPs were associated with aggressive PCa. Three of these 4 SNPs (rs9400968, rs1321366, and rs617426) were located at *RFX6*. A recent study reported that rs339331 could affect PCa risk by altering RFX6 expression, and suppression of RFX6 reduced PCa cell proliferation, migration, and invasion[21]. The association between rs1606365, located at *GPRC6A,* and aggressive PCa further suggests that *GPRC6A* may also be involved in PCa migration and invasion.

Ligands and activators of GPRC6A, including amino acids, calcium, and osteocalcin, have been reported to play a role in PCa pathogenesis[22-24]. Osteocalcin released into the circulation by activated osteoblasts is used as a marker and therapeutic target of PCa metastasis[25]. Other studies have shown that calcium and osteocalcin could induce the chemotaxis of PCa cells expressing *GPRC6A*[13]. Moreover, Karsenty et al[26] conducted a literature review and proposed that osteocalcin likely mediates interactions between bone and testicular cells those expressing *GPRC6A*. Therefore, we hypothesized that GPRC6A, as a receptor of osteocalcin, may be involved in the bone metastasis of PCa by mediating the crosstalk between bone and PCa cells. Indeed, in this report, we found that the GPRC6A expression in bone-derived PC3 and VCaP cells was higher than that in both normal prostate RWPE-1 cells and lymph node-derived Lncap cells.

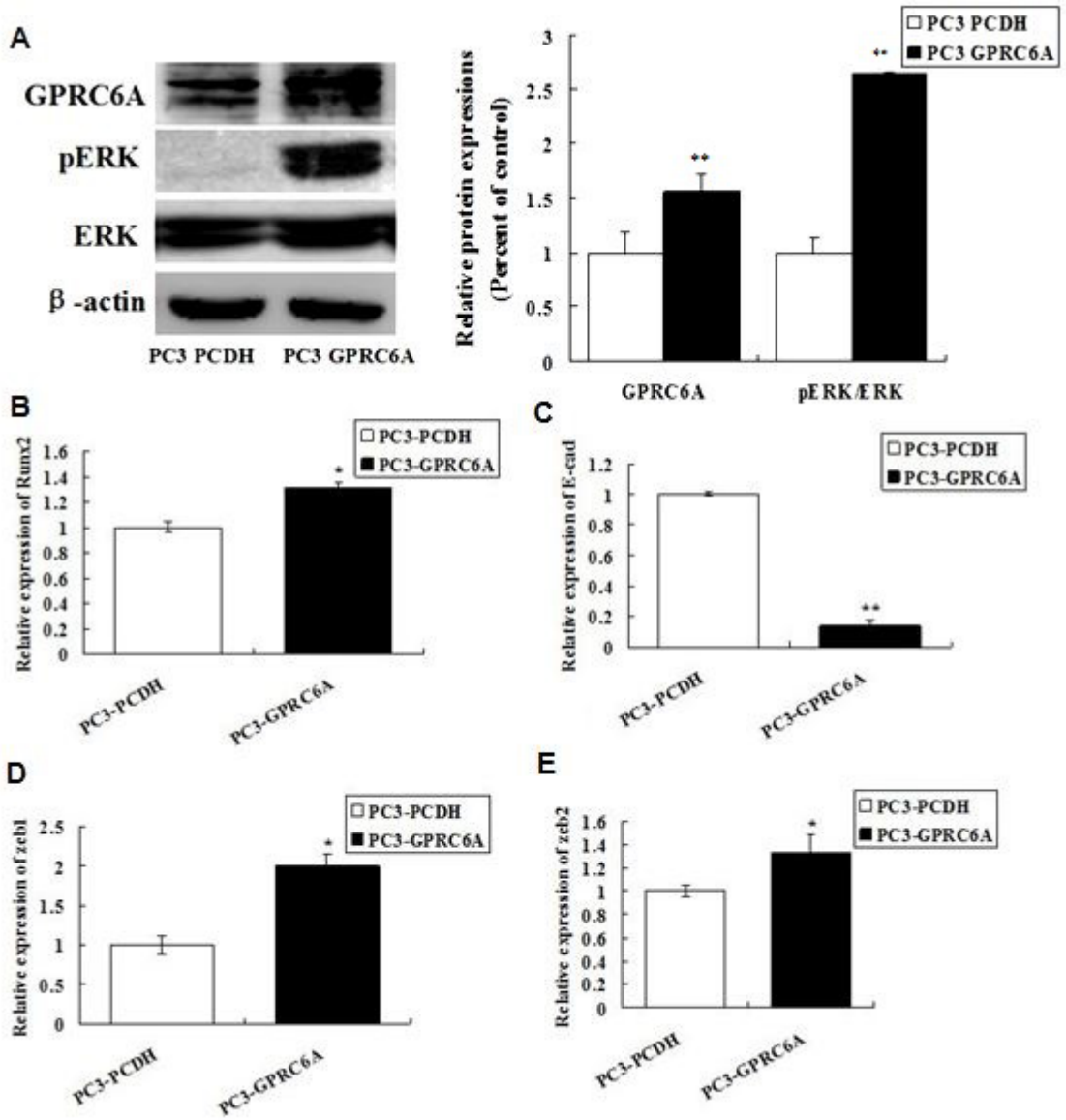

Figure 6. Expression of ERK signaling and EMT markers in GPRC6A overexpression PCa cells. The expression of pERK was significantly increased in the GPRC6A overexpression PCa cells (PC3-GPRC6A) compared with the control cells infected by the lentivirus with empty vector (PC3-PCDH) (A) The expressions of RUNX2, ZEB1 and ZEB2 in PC3-GPRC6A cells were higher than those in PC3-PCDH cells (B, D, E). The expression of E-cad was significantly reduced in the GPRC6A overexpression PCa cells compared with control cells. $^{*}p < 0.05$, *versus* control group. $^{**}p < 0.01$, *versus* control group.

The first step of PCa metastasis is tumor growth, followed by detachment of cancer cells and invasion of the prostate stroma[27]. In our study, we observed a phenotypic change of cell migration and invasion behavior in PC3 cells following GPRC6A knockdown, suggesting that the migration and invasion abilities could be reduced if PC3 cells are treated with *GPRC6A* siRNA. The scratch test and transwell migration assay results also suggested that the migration of PC3 cells was reduced following *GPRC6A siRNA* transfection. It is well known that EMT is an important phenotype during cancer metastasis[19]. A meta-analysis reported the reduced E-cadherin expression is associated with gastric cancer metastasis in Asian patients[28]. In our study, the expression of E-cad was reduced, and ZEB was increased in GPRC6A overexpression PC3 cells, which would promote the detachment and metastasis of PCa cells.

Tumor invasion and subsequent metastasis rely on the degradation of the ECM, which can be facilitated by the proteases MMP2 and MMP9[29].

In our study, the expression levels of MMP2 and MMP9 in all four-cell lines and the GPRC6A-knockdown PC3 cells tended to match with the pattern of GPRC6A expression. Moreover, gelatin zymography demonstrated that the enzymatic activities of MMP2 and MMP9 in the cell culture medium secreted by PCa cells were also reduced from treatment with *GPRC6A* siRNA. GPRC6A is known to be a potent activator of ERK signaling, and expression of pERK has been demonstrated to be higher in PCa cells with ligand-induced GPRC6A overexpression[13,24]. In our study, the expression of pERK was significantly increased in GPRC6A overexpression PC3 cells compared with that in control PC3 cells. Several tumor progression-associated genes have been shown to promote the migration and invasion of tumor cells by regulating the expression of MMPs via activation of the ERK signaling pathway[30,31].

PCa metastasis is a complex pathological process. In view of the literature described above combined with our present findings, we propose the following hypothesis for the involvement of GPRC6A in PCa bone metastasis. First, increased expression of GPRC6A may alter the migration and invasion abilities of tumor cells by activating the ERK pathway (possibly via the tumor metastasis-associated signaling Ras/RAF/MEK/ERK pathways, EMT, and MMPs expression). Second, osteocalcin in the blood circulation and bone is released from osteoblasts to exert a chemotactic effect to the tumor cells expressing GPRC6A. Third, tumor cells may interact with bone cells expressing GPRC6A: growth factors secreted from tumor cells expressing GPRC6A may promote osteoblastic activation, and the activated osteoblasts would secrete more osteocalcin, thus forming a vicious cycle. In this work, we have only focused on the first step to provide a simple confirmation that the metastatic phenotype in PCa cells is proportional to the expression level of GPRC6A. Therefore, to more comprehensively test the hypothesis described above and provide insight into the mechanism of aggressive PCa, more detailed studies in cell and animal models, as well as in patients with PCa bone metastasis, will be required.

Conclusions

Our data suggest that *GPRC6A* variants are associated with aggressive PCa and that inhibition of GPRC6A expression can decrease the migration and invasion of PCa cells. Further exploration of the molecular mechanism underlying the role of GPRC6A in PCa progression may offer a novel approach for predicting and treating human PCa bone metastasis.

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Conflicts of interest

The authors declare no conflicts of interest.

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