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FULL LENGTH ARTICLE

ARHGEF38 as a novel biomarker to predict aggressive prostate cancer

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Abstract Prostate cancer (PCa) metastasis is considered the leading cause of cancer death in males. Therapeutic strategies and diagnosis for stage-specific PCa have not been well understood. Rho guanine nucleotide exchange factor 38 (ARHGEF38) is related to tumor cell polarization and is frequently expressed in PCa. Microarray data of PCa were downloaded from GEO and TCGA databases. A total of 243 DEGs were screened, of which, 32 genes were upregulated. The results of enrichment analysis showed the participation of these DEGs in the tumor cell metastasis pathway. ARHGEF38 was significantly up-regulated in the four most prevalent cancers worldwide ($p < 0.05$), and its expression was higher in the tumor samples with higher Gleason score (GS). IHC, qRT-PCR, and western-blot analyses showed the higher expression of ARHGEF38 in PCa than benign prostatic hyperplasia (BPH). In addition, IHC results demonstrated a higher expression of ARHGEF38 in high-grade PCa than the low-grade PCa.

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Introduction

PCa is one of the most common types of cancer expected to occur in all males and accounts for 1 in 5 new diagnoses, excluding lung and bronchus, of the male patients; PCa is the second leading cause of cancer death in men in the United States.[1] Cancer metastasis is the primary cause of morbidity and mortality and is responsible for more than 90% cancer-associated deaths. Despite the advancement in treatment strategies over the past 30 years, early diagnosis of PCa to improve the prognosis has not been achieved.[2–4] Although the molecular alterations in PCa have been studied extensively, the specific molecular markers responsible for the metastasis and progression of PCa remain to be identified.[5]

The cell migration cycle is a highly regulated multistep process that begins with the membrane polarization and extension in the direction of migration.[6] The polarization of a cell is a key process for migration, which means that the direction of cell movement is determined by different molecular processes. The membrane protrusions establish contact with their environment through a diverse array of receptors via controlled adhesive interactions with the actin cytoskeleton and the extracellular matrix (ECM). Recent researches have focused on the main role of the focal adhesion complexes focal adhesions and adherens junctions, in epithelial cell behavior.[7–9]

The central role of the Rho family proteins is to regulate membrane protrusions to direct cell migration. Rho family small guanosine triphosphate (GTP) − binding proteins (GTPases) play a key role in cancer malignancy via regulation of multiple biological processes.[10] control the formation of cellular pseudopods. Rho GTPases act as pivotal molecular switches by alternating GTP activation and GDP inactivation, where the conversion of GDP to GTP is catalyzed by guanine nucleotide exchange factors (GEFs) in the Dbl family. Some of the Rho GEFs have been found to possess oncogenic functions playing a crucial role in the migratory process.[11–13] ARHGEF38 being a member of the Rho GEFs family might be of significant interest to study their cellular roles and mode of regulation in cancer.

Materials and methods

Gene screening

The gene expression array data sets were selected from the GEO public database. GSE21034 dataset with 126 PCa and 29 BPH tissues (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21034), GSE54808 dataset with 18 PCa and 12 normal prostate tissues (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54808), and TCGA dataset including 497 PCa tissues and 52 normal samples (http://gdac.broadinstitute.org/runs/stddata__2016_01_28/data/PRAD/20160128/) were used in this study. We changed all data to (log)2(ratio) format and then used BRB software to investigate mRNA expression of ARHGEF38 and further survival analyses were carried out.

Identification of DEGs

R limma package (version 3.4.2) and BRB software were used to identify DEGs ($p < 0.0001$) and calculate the fold change of their expression in PCa (|FC|>2.0). The DEGs were clustered using Consensus Clustering Plus and visualized using Java TrueView software. These DEGs were then uploaded to the web-based GEne SeT AnaLysis Toolkit database and KEGG to process for their function enrichment and pathway analyses. Finally, the copy number variations (CNVs) of DEGs were analyzed and the co-expression network was obtained.

Patient information and clinical specimens

Prostate tissue samples were obtained from patients with PCa ($n = 100$) and BPH ($n = 20$) who were treated at Second Hospital, Tianjin Medical University, between July 2013 and July 2014. The patients who had undergone radiotherapy or chemotherapy before surgery were excluded. This study was approved by the Institutional Research Ethics Committee, Second Hospital, Tianjin Medical University. Pathological diagnosis and clinical information (age, clinical stage, GS, lymph node metastasis, and differentiation) were determined by two pathologists. Based on the work of Borley,[14] a combination of pretreatment GS and clinical stage was used to divide the patients into normal, low-grade, intermediate-grade, high-grade, and aggressive groups. A case−control study of Chinese man with BPH and the five PCa groups were stratified according to WHO classification: (i) 20 BPH tissues; (ii) 20 low-grade GS 2−6 (LGPCa); (iii) 40 intermediate GS7 (MGPCa) (3 + 4 and 4 + 3); (iv) 20 high-grade GS8 (HGPCa); and (v) 20 aggressive GS9 and GS10.

Immunohistochemistry staining and scoring

Immunohistochemistry was performed to detect the protein expression of ARHGEF38 (ab122345, Abcam; polyclonal rabbit anti-human, 1:100) and Ki67 (#9949, CST; monoclonal mouse anti-human, 1:800) in 100 paraffin-embedded PCa tissues and 20 paraffin-embedded BPH tissues. Intensity and extent of staining were scored as follows: 3 (+++), strong staining (50%−100% positivity); 2 (++), medium staining (25%−50%); 1 (+) weak staining (10%−25%); (−) equivocal or absence of staining (<10%). IHC image processing was performed using Image Pro-plus 6.0 software.[15,16]

RNA extraction and qRT-PCR experiment

Total RNA was isolated from PCa and BPH tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. PrimeScript™ RT reagent Kit (Takara) was used for RNA transcription. The primers (Qing Ke Company) used in this study are presented in Table 1.

Western blot analysis

Total protein was extracted from the tissues using RIPA lysis buffer (P0013C, Beyotime) according to the manufacturer's

Table 1 List of primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Primer sequence
ARHGEF38	F: 5′-ACCTTGAGGAGGAGCCAATC-3′
	R: 5′-TCTTTTCCCGCTTTGCCATC-3′
GADPH	F: 5′-ACCTGACCTGCCGTCTAGAA-3′
	R: 5′-TCCACCACCCTGTTGCTGTA-3′

Abbreviation: F, forward; R, Reverse.

instructions. The tissue lysates were resolved on 10% SDS-PAGE gels, transferred to PVDF membrane, and then probed with anti-ARHGEF38 (ab122345, Abcam, 1:2000) and anti-beta actin (CAT#66009-1-Ig, Proteintech, 1:5000), followed by incubation with secondary antibodies (CAT#7074, CST, 1:2000). The proteins were finally detected using the SignalFire™ Plus ECL Reagent (CAT#12630, CST).

Statistical analyses

Mean and standard deviation (SD) were used to indicate the degree of dispersion of the data. Chi-square test was used to assess the significance of ARHGEF38 expression when grouped using mRNA expression, age, PSA, and GS. Pearson test was used for pathological grading, correlation analysis of GS, and ARHGEF38 expression. The survival analyses were performed using the R survival analysis package. The analyses were performed with respect to the expression of ARHGEF38 and Ki67. The statistical significance for the data was analyzed using SPSS 19.0 software. Two-tailed Student's *t*-test was used to compare two independent groups. $p < 0.05$ was considered statistically significant.

Results

Differential expression analysis and enrichment analysis

After analyzing the consistency of the data and standardization, a total of 243 DEGs were selected from 3 datasets, of which, 32 genes were upregulated and 211 genes were downregulated. Fold change values of at least 2.0 and $p < 0.001$ were regarded as significant (Fig. 1A). BPH and normal tissues served as controls. Enrichment analysis was

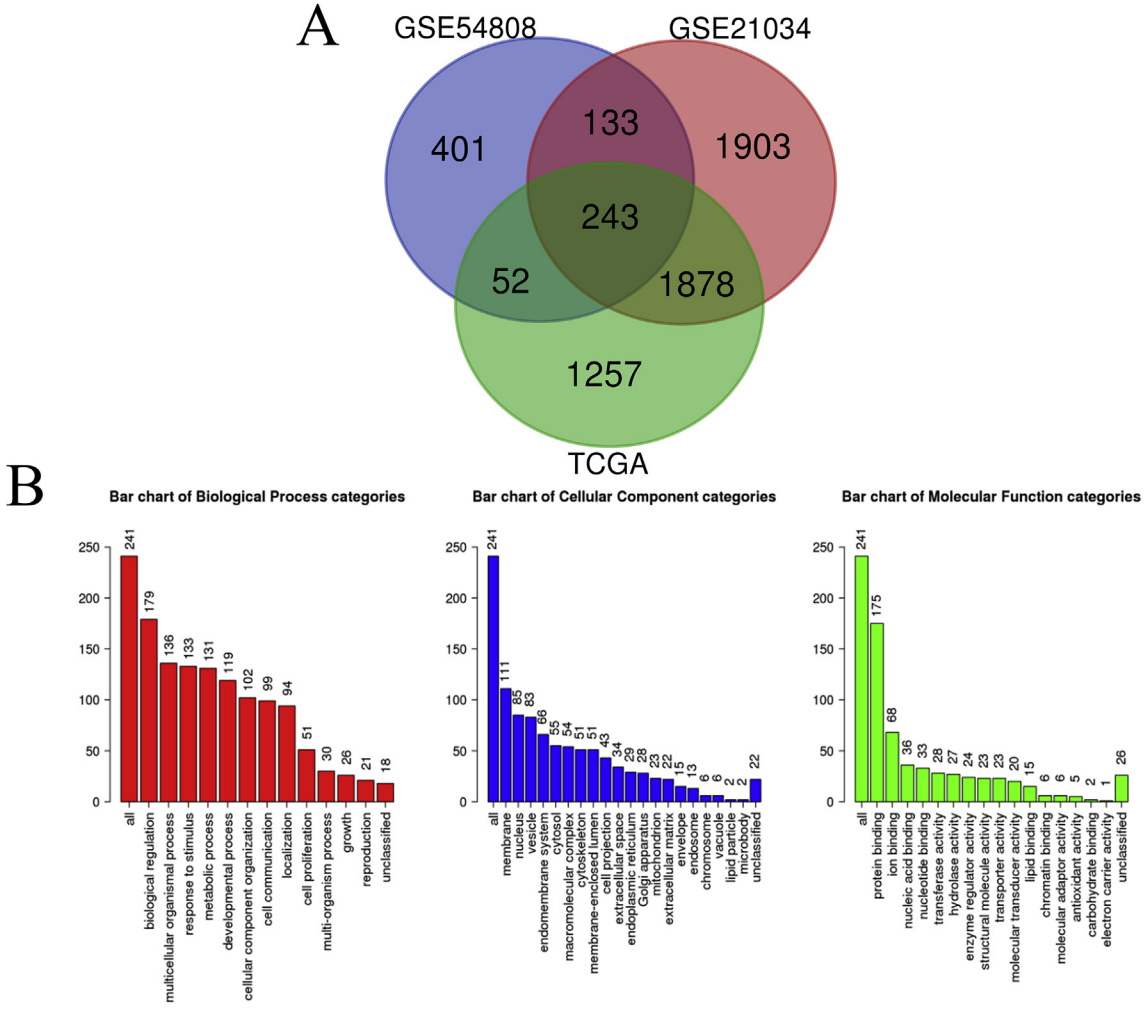

Figure 1 DEGs and enrichment analysis. **(A)** In total of 243 DEGs were selected from the 3 datasets, including 32 up-regulated genes and 211 down-regulated genes. **(B)** Enrichment analysis showed that the DEGs were related to biological regulation, multicellular organismal process and other biological processes (BP), membrane, nucleus, vesicle and cell components (CC), protein binding, ion binding, nucleic acid binding and molecular function (MF).

performed by WebGestaltR to further identify the functions of DEGs (Fig. 1B). The results indicated that the DEGs were related to biological regulation, multicellular organismal process, and biological processes in the cell such as protein binding, ion binding, nucleic acid binding, and other molecular functions.

ARHGEF38 expression is significantly increased in human PRAD

The primary aim of this study was to identify a diagnostic and prognostic biomarker for PCa. Two gene expression profile datasets (GSE21034 and GSE54808) from GEO database were selected.[17,18] Meanwhile, we also further verified the difference in mRNA expression level of the ARHGEF38 gene in PRAD in TCGA datasets. The differential expression analysis of ARHGEF38 using the data obtained from GEO and TCGA databases are summarized in Table 2. BPH and normal tissues were considered as the control group (N), while PCa tissues were regarded as the experimental group (C). Furthermore, the expression of ARHGEF38 in six cancers colon adenocarcinoma (COAD), lung squamous cell carcinoma (LUSC), PRAD, kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), and bladder urothelial carcinoma (BLCA) were analyzed using TCGA and Genotype-Tissue Expression (GTEx) sequencing data. The results showed that ARHGEF38 and Ki67 were upregulated in COAD, PRAD, KICH, and BLCA tissues (Fig. 2A and D). Meanwhile, compared to normal tissues, the expression of ARHGEF38 and Ki67 were significantly increased in PRAD (Fig. 2B and E). The expression of ARHGEF38 mRNA in the GS8 group was significantly higher than that in the GS6 and GS7 groups (Fig. 2C). Furthermore, A positive correlation between ARHGEF38 expression and Ki67 expression in TCGA PRAD tumor data set indicated the increase in ARHGEF38 expression with an increase in proliferation ($r = 0.443$, Fig. 2F).

Table 2 Differential expression analysis of ARHGEF38 in PRAD in GEO and TCGA databases.

Gene symbol	GSE21034 (C = 126, n = 29)		GSE54808 (C = 18, n = 12)		TCGA (C = 499, n = 52)	
	FC	p Value	FC	p Value	FC	p Value
ARHGEF38	4.538	1.45E-15	2.9	1.68 E-4	2.56	1.11E-27

In bold are significant differences (*$p < 0.05$). BPH and normal tissues were considered as control group (N), while PCa tissues were regarded as experimental group (C).

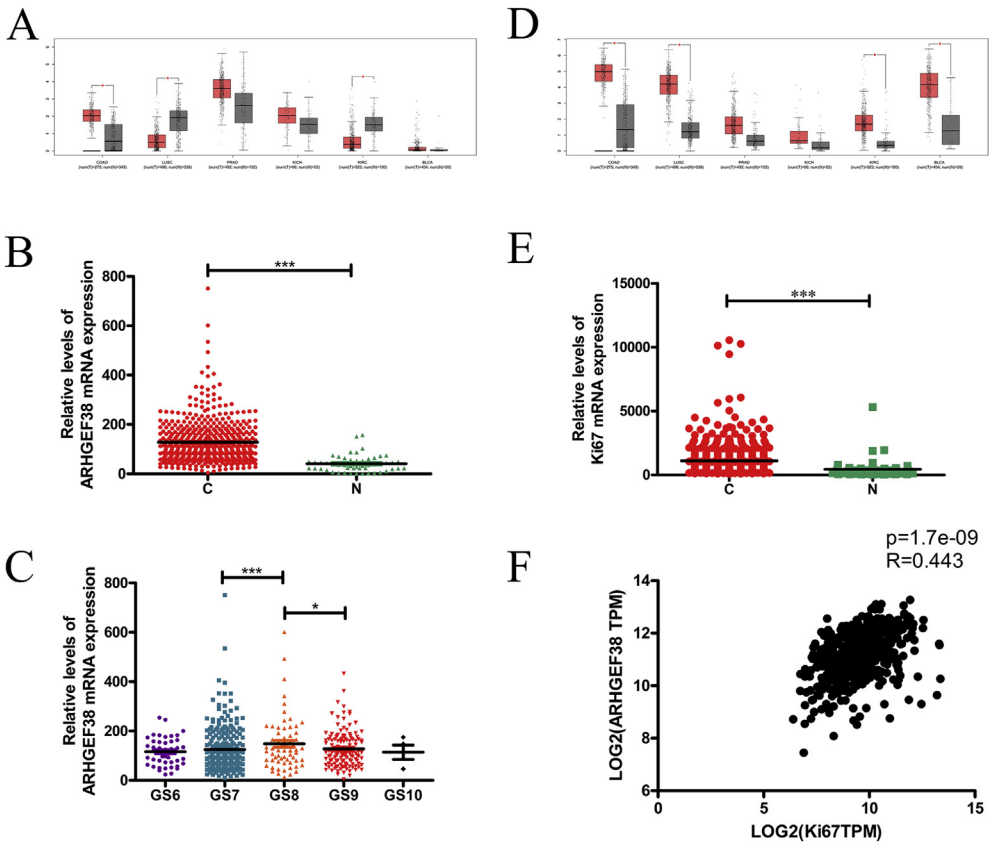

Figure 2 ARHGEF38 and Ki67 is overexpressed in PCa tissues using TCGA and GTEx sequencing data. **(A)** ARHGEF38 expression levels in COAD, LUSC, PRAD, KICH, KIRC and BLCA. **(B)** ARHGEF38 expression was analyzed by RNA-seq in PCa ($n = 499$) and normal tissues ($n = 52$). **(C)** ARHGEF38 expression in PRAD by GS categories. **(D)** Ki67 expression levels in COAD, LUSC, PRAD, KICH, KIRC and BLCA. **(E)** Ki67 expression was analyzed by RNA-seq in PCa ($n = 499$) and normal tissues ($n = 52$). **(F)** The correlation between ARHGEF38 and ki67 expression was detected by analyzing TCGA data set.

IHC studies to determine the expression of ARHGEF38 and Ki67 in PCa

IHC was performed to detect the expression of ARHGEF38 and Ki67 (encodes a nuclear protein that is associated with and may be necessary for cellular proliferation), in differential grade tumors (Fig. 3). The results indicated that the combined detection of ARHGEF38 and Ki67 in the PCa tissue samples could easily differentiate the tumors according to their grade. We observed that ARHGEF38 was not expressed in BPH tissues (Fig. 3A). Meanwhile, its expression intensity in HGPCa (Fig. 3D) was significantly higher than in MGPCa (Fig. 3C) and LGPCa (Fig. 3B). Ki67 was weakly expressed in BPH (Fig. 3E), but the expression of Ki67 enhanced with the increase in GS score of the PCa samples (Fig. 3F–H). Consistent with the previous study by Rebecca L[1] on the age-based grouping of the patients with invasive cancer in the US population, the protein expression of ARHGEF38 in patients older than 68 years was significantly higher than that in patients below 68 years (Table 3).

Expression of ARHGEF38 and Ki67 in tissues and survival analysis

mRNA expression of ARHGEF38 in PCa was significantly higher than that in BPH ($p < 0.0001$, Fig. 4A). Similarly, expression of the ARHGEF38 protein in PCa was also significantly higher than that in BPH (Fig. 4B). Quantitative analysis of the IHC results showed that the staining intensity was significantly higher in the metastasis group than in the non-metastasis group ($p < 0.0001$, Fig. 4C). This indicates that the ARHGEF38 expression is proportional to the aggressiveness of PCa. The intensity of ARHGEF38 protein staining in patients with stage 3 PCa was significantly higher than that in stage 2 patients ($p < 0.0001$, Fig. 4D). The rate of ki67 positive cells in patients with T3 PCa was

Table 3 Relationship between ARHGEF38 expression and clinicopathological factors.

characteristic	ARHGEF38		*p*-value
	High expression (stain50-100)	Low + intermediate expression (stain<50)	
Age (year)			&<0.0001
68 ± 16.2			
≥68	41	16	
<68	8	35	
PSA (ng/ml)			&<0.0001
≥10	38	15	
<10	10	37	
Pathology stage			#<0.0001
T1	2	9	
T2	18	37	
T3	28	5	
T4	1	0	
Gleason score			#<0.0001
≤6	5	15	
7	12	28	
8	12	8	
9,10	20	0	

& Chi-square test, two-sided(***$p < 0.001$).
# Pearson test(***$p < 0.001$).

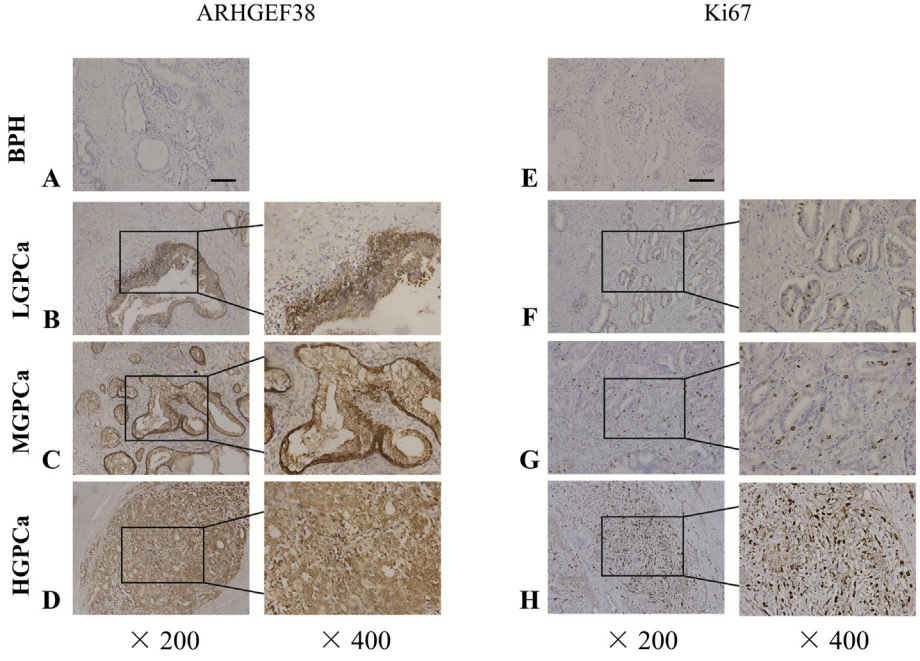

Figure 3 Comparison of ARHGEF38 and Ki67 expression in BPH, LGPCA, MGPCa and HGPCA. **(A)** ARHGEF38 was not expressed in BPH tissues. Meanwhile, its expression intensity in **(D)** HGPCa was significantly higher than in **(C)** MGPCa and **(B)** LGPCa. **(E)** Ki67 was weakly expressed in BPH, but the expression of **(F–H)** Ki67 enhanced with the increase in GS score of the PCa samples.

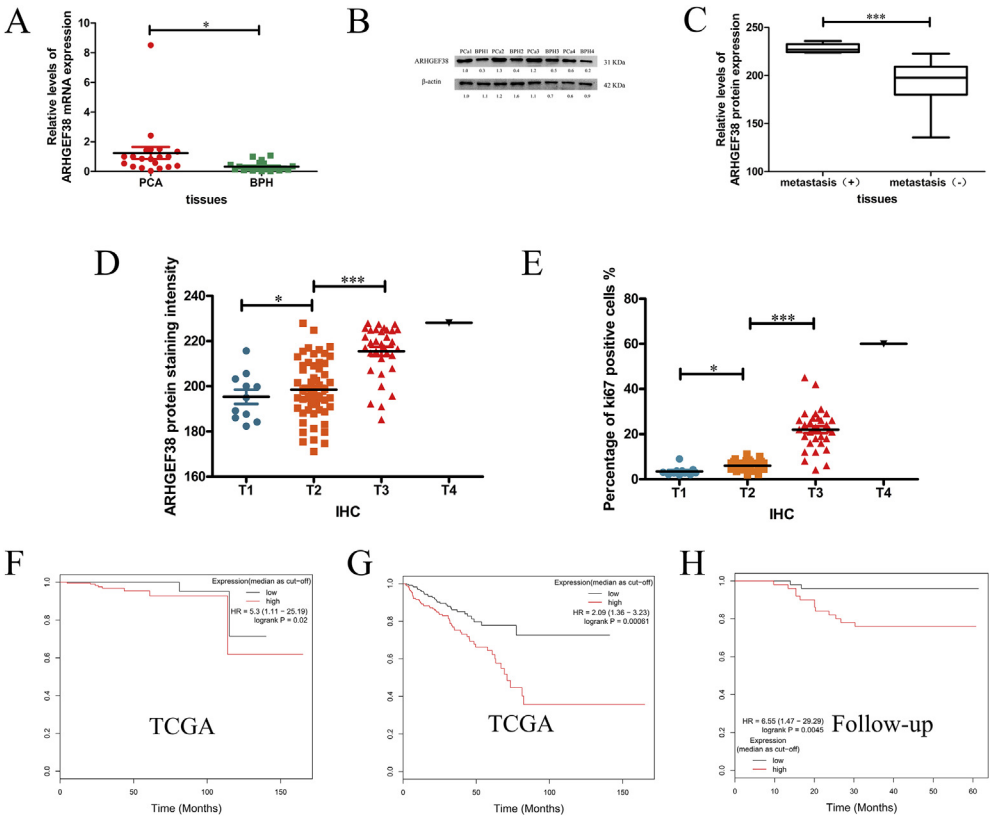

Figure 4 The expression of ARHGEF38 in PCa tissues. **(A)** The expression of ARHGEF38 mRNA in PCa was significantly higher than that in BPH (***$p < 0.0001$). **(B)** The expression of ARHGEF38 protein in PCa was significantly higher than BPH. **(C)** The expression of ARHGEF38 protein in PCa metastatic samples was significantly higher than that of non-metastatic samples. **(D)** The expression of ARHGEF38 protein in stage 3 (T3) was significantly higher than stage 2 (T2) (***$p < 0.0001$). **(E)** The rate of ki67 positive cells in patients with T3 PCa was significantly higher than that in patients with T2 (***$p < 0.0001$). **(F)** Kaplan–Meier curves for test series (low = 249, high = 250) *$p = 0.02$, HR = 5.3. **(G)** Kaplan–Meier curves for test series (low = 249, high = 250) *$p = 0.00061$, HR = 2.09. **(H)** Kaplan–Meier curves for test series (low = 50, high = 50) **$p = 0.0045$, HR = 6.55.

significantly higher than that in patients with T2 ($p < 0.0001$, Fig. 4E). According to the survival curves, we observed that the survival rate of patients with high expression of the ARHGEF38 gene was significantly lower than those with low expression in the TCGA PRAD tumor dataset (high = 250, low = 249, $p = 0.02$, HR = 5.3) (Fig. 4F). Similarly, the survival rate of patients with higher expression of ki67 is accompanied by poorer prognosis (high = 250, low = 249, $p = 0.00061$, HR = 2.09) (Fig. 4G). Our follow-up results for 100 patients with PCa also indicated that the prognosis of patients was dependent on the ARHGEF38 expression (high = 50, low = 50, $p = 0.0045$, HR = 6.55) (Fig. 4H). These results strongly suggest that ARHGEF38 might be a potential biomarker candidate to predict PCa prognosis.

Discussion

With the advancement in sequencing technologies over the last decade, gene expression analysis has become far much accessible for oncological research. Various strategies have been applied to identify diagnostic and prognostic markers based on the gene expression profiles from TCGA and GEO datasets. One approach is to analyze the known gene interaction networks and pathways to predict the progression and survival of cancer patients. Previous studies have shown that the RAS superfamily genes Cdc42, RAC, and RHOA, play an important role in tumorigenesis and metastasis, and have been implicated in cancer prognosis.[19–22] RHOGEF catalyzes the conversion between GTP and GDP, thereby modulating CDC42, RAS, and RHOA activities involved in the regulation of various cellular processes related to cancer cells proliferation.[23,24]

In this study, we retrieved the microarray data from GEO and TCGA and identified 243 DEGs through different bioinformatics methods. Among the identified DEGs, 32 genes were upregulated and 211 were downregulated. These DEGs were found to be the genes involved in biological processes such as muscle movement, cell migration, and actin filament-based process. Hence, we suggest that the DEGs might be involved in tumor metastasis processes. Among the DEGs, CDC42EP3, ABCC9, FGFR2, PRDM5, and ARHGEF38 were found to be co-expressed. In particular, ARHGEF38 was significantly upregulated in PCa samples analyzed using GEO and TCGA PRAD data sets. Analyzing the TCGA dataset indicated that ARHGEF38 was not only overexpressed in PRAD, but also in the five most prevalent

cancers except breast cancer in the United States. In addition, the analysis of RNA-seq PRAD data from the TCGA dataset indicated that ARHGEF38 is significantly upregulated in PCa compared to normal prostate tissue and the ARHGEF38 expression positively correlated with the degree of cancer progression.

ARHGEF38 expression levels were confirmed using the PCa samples from patients. We observed that ARHGEF38 was increased in the PCa tissues both at the mRNA and protein levels. The results from the present study demonstrated that ARHGEF38 is significantly overexpressed in PCa than BPH, especially, high-grade prostate cancer. This suggested that ARHGEF38 might promote prostate cancer migration and hence attribute to PCa progression. We also analyzed the association between ARHGEF38 expression and clinicopathological features in PCa patients. The results suggested that the ARHGEF38 expression was positively correlated with GS and the pathology stage. Solid tumor progression is characterized by metastasis to regional lymph nodes and dissemination to distant organs. Lymph node metastases in cancer patients are associated with tumor aggressiveness and poorer prognoses.[25–27] ARHGEF38 protein in lymph node metastasis patients was significantly higher than that in the non-metastatic patients, which may suggest that the high expression of ARHGEF38 is more prone to distant metastasis; thus, ARHGEF38 could be an indicator for PCa metastasis. Therapeutic strategies and diagnosis for stage-specific PCa have not been well understood. In this study, ARHGEF38 and Ki67 can clearly distinguish T1, T2 and T3 from PCa, providing useful support for further treatment strategies. Ki67 is a well-known proliferation marker used in pathological grading. It has been reported to predict the clinical outcome in prostate cancer.[28,29] Our results showed that the expression of the Ki67 protein was high in HGPCa similar to the previous report by Lynn et al.[30] We also demonstrated a positive correlation between Ki67 and ARHGEF38.

The Kaplan Meier Survival analysis showed lower survival rates with an increased expression of ARHGEF38 and Ki67. The probability of 5-year tumor-free survival of patients with low ARHGEF38 expression was 5.3 times (TCGA) and 6.5 times (follow-up patients) higher than the patients with high ARHGEF38 expression. Similarly, the probability of 5-year tumor-free survival of patients with low Ki67 expression was 2.09 times higher than the patients with high Ki67 expression. Collectively, the present study hints a novel biomarker to detect PCa prognosis, which might help to design better treatment strategies for better survival rates of PCa patients.

Conclusion

The present study unravels the differentially expressed genes in PRAD from GEO datasets, which were further verified using TCGA data. The functional enrichment analysis indicated that most of the DEGs were related to metastasis. Through IHC and qRT-PCR analyses, we found that expression of ARHGEF38 in high-grade prostate cancer was higher than that in low-grade prostate cancer, especially in GS8 patients, and the expression was higher than that in GS7 and GS2-6 patients. The expression of ARHGEF38 in patients with lymph node metastasis was higher than in the non-lymph node metastasis patients. We also found a positive correlation between Ki67 and ARHGEF38 in prostate cancer. The higher expression of both worsened the prognosis. Taken together, our results reveal that ARHGEF38 might play a crucial role in tumorigenesis and metastasis of human PCa and might function as a potential prognostic indicator of PCa. This might further shed light on to the development of therapeutic strategies according to the aggressiveness of PCa by allowing early diagnosis and prognosis of PCa.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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