SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 5450-5458, 2017

Significance of the *TMPRSS2:ERG* gene fusion in prostate cancer

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Received November 4, 2016; Accepted June 28, 2017

DOI: 10.3892/mmr.2017.7281

Abstract. The transmembrane protease serine 2:v-ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2:ERG)* gene fusion is common in prostate cancer, while its functional role is not fully understood. The present study aimed to investigate the significance of the *TMPRSS2:ERG* gene fusion in human prostate cancers using bioinformatics tools. Comprehensive alteration analysis of *TMPRSS2* and *ERG* in 148 different human cancer studies was performed by cBioPortal, and the mRNA expression level of the *ERG* gene was evaluated using Oncomine analysis. Furthermore, lentiviral short hairpin (sh)RNA-mediated knockdown of *TMPRSS2:ERG* was performed to study the impact of *ERG* silencing on cell proliferation and cell cycle distribution in prostate cancer cells. The results demonstrated that the *TMPRSS2* and *ERG* genes were mostly altered in prostate cancer, and the most frequent alteration was gene fusion. Oncomine analysis demonstrated that the *ERG* gene was significantly upregulated in prostate clinical samples compared with the normal prostate gland in four independent datasets, and a positive association was observed between potassium inwardly-rectifying channel subfamily J member 15, down syndrome critical region gene 4, potassium inwardly-rectifying channel subfamily J member 6 and *ERG* gene expression. There were 272 mutations of the *ERG* gene identified in the cBioPortal database; among the mutations, 2 missense mutations (R367C and P401H) were regarded as functional mutations (functional impact score >1.938). Furthermore, the present study successfully knocked down *ERG* gene expression through a lentiviral-mediated gene silencing approach in VCaP prostate cancer cells. The *ERG* mRNA and protein expression levels were both suppressed significantly, and a cell-cycle arrest at G_0/G_1 phase was observed after *ERG* gene silencing. In conclusion, these bioinformatics analyses provide novel insights for *TMPRSS2:ERG* fusion gene study in prostate cancer. Target inhibition of *ERG* expression could significantly cause cell growth arrest in prostate cancer cells, which could be a potentially valuable target for prostate cancer treatment. However, the precise mechanism of these results remains unclear; therefore, further studies are required.

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Key words: prostate cancer, transmembrane protease serine 2, v-ets erythroblastosis virus E26 oncogene homolog, fusion gene, bioinformatics

Introduction

Prostate cancer is one of the most frequent malignancies and the most common leading cause of cancer-associated death in men all over the world, particularly in developed countries (1,2). In the past decades, prostate specific antigen (PSA) was the only widely-used serum biomarker for prostate cancer. However, due to the extensive use of serum PSA testing, the prostate cancer-specific mortality has increased significantly, which results in over-diagnosis or over-treatment (3). Multiple technologies have been applied to identify novel prostate cancer biomarkers in tissues and blood of patients. Nevertheless, no biomarker has been identified to replace the routine use of PSA at present.

Recently, gene fusion transcripts of transmembrane protease serine 2 (*TMPRSS2*):v-ets erythroblastosis virus E26 oncogene homolog (*ERG*), also termed *TMPRSS2:ERG* or *T2E*, have been identified as promising urinary novel biomarkers in prostate cancer (4,5). A study in 2005 demonstrated that up to 55% prostate cancer cases were identified to have *ERG* over-expression, using a novel biostatistical method called cancer outlier profile analysis (6). Furthermore, the overexpression of *ERG* is in the majority of tumors driven by fusion of the *ERG* gene with *TMPRSS2*, which are both located on chromosome 21 (7). *TMPRSS2* is a prostate-specific and androgen-response gene that encodes a protein belonging to the serine protease family, which functions in prostate carcinogenesis and relies on gene fusion with ETS transcription factors, such as *ERG* and *ETV1* (8). *ERG* is an oncogene that encodes a member of the erythroblast transformation-specific family of transcription factors (9), which is a key regulator of

cell proliferation, differentiation, angiogenesis, inflammation and apoptosis. The *TMPRSS2:ERG* gene fusion is the most frequent genomic alteration in prostate cancer cases and results in overexpression of the transcription factor *ERG* (10), which is present in both early- and late-stage prostate cancer (castration-resistant prostate cancer, CRPC) (6,11).

Numerous studies have evaluated the significance of *TMPRSS2-ERG* in prostate cancer patients with varying results (12), some of which indicated that the fusion gene is not an important predicator of prostate cancer mortality and recurrence (13), while other studies demonstrated that *TMPRSS2:ERG* fusion was associated with an increased risk of prostate cancer mortality (13-16). The present study examined the expression pattern of the *TMPRSS2:ERG* fusion gene in human pan-cancers, including prostate cancer, by using the publically available data from cBioPortal. Based on these findings, the present study specifically analyzed the *ERG* alterations, mRNA expression, mutations and interaction networks in several prostate cancer datasets. Furthermore, the functional role of *ERG* in prostate cancer cells was examined by lentiviral-mediated knockdown approaches. The present study provides novel insights for the *TMPRSS2:ERG* fusion gene study in prostate cancer.

Materials and methods

Determination of TMPRSS2 and ERG alterations across different cancer types. The frequency of *TMPRSS2* and *ERG* gene alterations (including mutations, deletions, copy number gains and amplifications) was performed across multiple cancer types using the cBioPortal for Cancer Genomics database (www.cbioportal.org), which contains 147 common cancer studies with the details of almost 23,000 patients. All searches were performed according to the online instructions of cBioPortal.

Oncomine database analysis. *ERG* mRNA expression levels in prostate cancer were compared with its matched normal tissues by using The Cancer Genome Atlas (TCGA) datasets in the Oncomine database (www.oncomine.org). The threshold used to obtain the most significant probes of the queried gene for each microarray data included a two-fold difference in expression between cancers and normal tissues if $P<1x10^{-4}$. The mRNA expression level of *ERG* was analyzed in three independent datasets.

ERG gene silencing by short hairpin (sh)RNA in VCaP cells. The prostate cancer cell line VCaP which obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Three pairs of shRNAs for *ERG* (GenBank ID: NM_001136154.1) were designed (Table I), synthesized and packaged by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). shRNAs were cloned into a pLKO.1 puro vector (Addgene, Inc., Cambridge, MA, USA) according to the manufacturer's protocol. Lentiviral particles were generated following transfection of 80% confluent 293T cells (Type Culture Collection of the Chinese Academy of Sciences), with 15 μg pLKO.1-shRNA-ERG plasmid or empty control vector using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific) for 10 min at room temperature according to the manufacturer's instructions. VCaP cells were initially seeded at a density of $5x10^6$ cells/100 mm dish. After 24 h incubation, cultures were supplemented with $1x10^8$ lentiviral particles (multiplicity of infection of 8) with 8 μg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 48 h. Subsequently, total cell RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and DNaseI (New England BioLabs, Inc., Ipswich, MA, USA). Total RNA was reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. The temperature protocol that was used was as follows: At 37˚C for 15 min and at 85˚C for 5 sec. Quantitative polymerase chain reaction (qPCR) was used to evaluate the *ERG* silencing effect at the mRNA level using SYBR Premix Ex Taq (Takara Bio, Inc.) according to the manufacturer's instructions. The thermocycling conditions that were used were as follows: At 95˚C for 30 sec, followed by 40 cycles at 95˚C for 15 sec, at 60˚C for 20 sec and at 72˚C for 30 sec. The following oligonucleotide primers were used: ERG, forward 5'-ATCGCATTATGGCCAGCACT-3', reverse 5'-TGTCCATAGTCGCTGGAGGA-3'; and β-actin, forward 5'-GGACTTCGAGCAAGAGATGG-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3'. The relative gene expression data were assayed using the comparative Cq method as described previously (17,18).

Total cellular proteins were extracted from 70-80% confluent cultured cells after 48 h transfection using ice cold lysis buffer (20 mM HEPES, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 10 mM monothioglycerol, 1 mM PMSF, 5 mM leupeptin, 0.25 M sucrose). Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of extracted protein samples (30 μg) were separated by standard 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Folowing blocking with 5% non-fat dry milk at room temperature for 1 h, membranes were probed with optimally diluted primary antibodies at 4˚C overnight, then incubated with a horseradish peroxidase-conjugated secondary antibody (cat no. ab6721; 1:5,000; Abcam, Cambridge, MA, USA) at room temperature for 1 h. Protein bands were visualized with enhanced chemiluminescence western blot reagents (GE Healthcare Life Sciences, Little Chalfont, UK) as described previously (19). Blots were semi-quantified by densitometry using ImageJ software version 2.0 (National Institutes of Health, Bethesda, MD, USA). Primary antibodies used were as follows: Anti-ERG monoclonal antibody (cat no. ab92513; Abcam, Cambridge, MA, USA; 1:1,000) and anti-β-actin (cat no. 4970; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000).

Cell proliferation assay. Cell proliferation was assessed by MTT assay according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). The formed formazan crystals were dissolved by adding 100 μl/well acidic SDS buffer [10% SDS, 0.16% (6 mol/l) HCl and 5% isobutyl alcohol] and incubating overnight in a CO_2-free incubator at 37˚C. The optical density (OD)570 absorption was measured in a microplate reader (Perkin Elmer Victor3 1420 Multilabel Plate Counter, PerkinElmer, Inc., Waltham,

Table I. shRNA sequences targeting the *ERG* gene.

shRNA duplex	Sequence (5'-3')
ERG-shRNA1	
Forward	CCGGTGCTCATATCAAGGAAGCCTTATCAAGAGTAGGCTTCCTTGATATGAGCTTTTT
Reverse	AATTAAAAAGCTCATATCAAGGAAGCCTTACTCTTGAATAGGCTTCCTTGATATGAGC
ERG shRNA2	
Forward	CCGGTCCACCCACAGAAGATGAACTTTTCAAGAGAAGTTCATCTTCTGTGGGTGGTTTTT
Reverse	AATTAAAAACCACCCACAGAAGATGAACTTCTCTTGAAAAGTTCATCTTCTGTGGGTGG
EEG-shRNA3	
Forward	CCGGTGATGATGTTGATAAAGCCTTATTCAAGAGTAAGGCTTTATCACATCATCTTTTT
Reverse	AATTAAAAAGATGATGTTGATAAAGCCTTACTCTTGAATAAGGCTTTATCACATCATC

ERG, v-ets erythroblastosis virus E26 oncogene homolog; shRNA, short hairpin RNA.

MA, USA). Experiments were repeated three times, and data were represented as the mean of five-replicate wells ± standard error.

Cell cycle analysis. Cell cycle analysis of control and *ERG*-silenced VCaP cells from 3 independent biological replicates were collected. The cells were washed in PBS, and then fixed in 70% ethanol for 30 min at -20˚C. The fixed cells were washed three times, resuspended in PBS containing 10 μg/ml of RNase A for 30 min, and then incubated with 10 μg/ml propidium iodide (PI) for 30 min in the dark. Subsequently, the samples were used for DNA flow cytometry (ALTRA cell sorting system, Beckman Coulter, Inc., Brea, CA, USA) analysis. For each measurement, at least 15,000 cells were acquired. Analysis of cell cycle was performed with ModFit LT2 software version 2.0 (Verity Software House, Inc., Topsham, ME, USA).

STRING analysis. STRING software (https://string-db.org/) (20) was used to generate the network of predicted associations for ERG protein. The network was set in evidence mode, in which the associations of the proteins were predicted based on up to 7 different evidences (the presence of fusion evidence, neighborhood evidence, co-occurrence evidence, experimental evidence, text-mining evidence, database evidence and co-expression evidence).

Statistical analysis. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses. All analysis was performed using the unpaired Student's t-test or analysis of variance followed by a post hoc Tukey test for multiple comparisons. The data were presented as mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Determination of TMPRSS2 and ERG gene alterations across different human cancer types. By pan-cancer analysis, it was demonstrated that the *TMPRSS2* and *ERG* alterations (including mutations, deletions and amplifications) were mainly observed in one breast cancer study and most of the prostate cancer studies (Fig. 1A). In the breast cancer xenografts study (21) *TMPRSS2* and *ERG* were notably altered in 51.7% of 29 cases, among which 48.3% (14 cases) were amplification. Only one case contained a deep deletion in *ERG* gene in the breast cancer study mentioned above.

However, in prostate cancer studies, the most frequent alteration of *TMPRSS2* and *ERG* was gene fusion (Fig. 1B). Amplification, missense mutation and deep deletion were less frequently observed. Studies in prostate adenocarcinoma (22,23) showed that *TMPRSS*2 and *ERG* were altered in over 47% in prostate cancers, and 46% of them were gene fusion. Moreover, two metastatic prostate cancer datasets (24,25) demonstrated that 42 and 49% of the patients had *ERG* gene fusion (Fig. 1B).

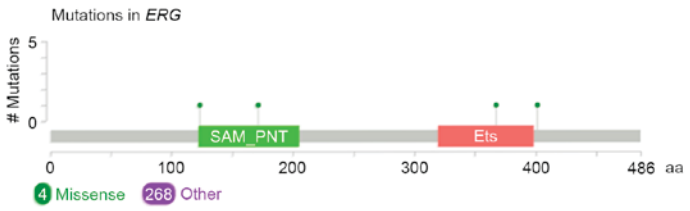
Although there was less frequent mutation than gene fusion observed in *TMPRSS2* and *ERG*, some of them may serve important roles in prostate cancer progression. There were 272 mutations of the *ERG* gene identified in the cBioPortal database; among the mutations, 2 missense mutations (R367C and P401H) were regarded as functional mutations (Functional impact score >1.938). The details of missense mutations of *TMPRSS2* and *ERG* with high mutation assessor score are presented in Fig. 2A and B.

ERG gene is overexpressed in prostate cancer clinical samples. Given the high-frequent alterations of *TMPRSS2* and *ERG* observed in prostate cancer studies, the mRNA expression profile of *ERG* in prostate cancer in four independent datasets were analyzed using Oncomine analysis. Notably, *ERG* mRNA expression levels were significantly upregulated in prostate cancer cases compared with their normal tissues in all four independent datasets (Fig. 2C). Furthermore, the co-expression gene of *ERG* in a cohort of 230 patients with prostate cancer was also evaluated (Taylor Prostate) (26) in the Oncomine database, as well as the interaction networks by STRING (Fig. 3B). The most correlated gene of *ERG* was potassium inwardly-rectifying channel subfamily J member 6 (*KCNJ6*), potassium inwardly-rectifying channel subfamily J member 15 (*KCNJ15*) and down syndrome critical region gene 4 (*DSCR4*; Fig. 3A).

Figure 1. Alteration frequency analysis of *TMPRSS2* and *ERG* gene in human cancers using cBioPortal. (A) The alteration frequencies of *TMPRSS2* and *ERG* across 148 cancer studies (min. % altered samples: 2% are presented). Gene amplification is the highest in breast cancer while gene fusion is the most frequent in prostate cancers. The red bars indicate gene amplification, blue bars are homozygous deletions, green bars are non-synonymous mutations, and gray bars indicate multiple alterations. (B) Gene fusion frequencies of *TMPRSS2* and *ERG* in prostate cancers in four independent studies. Purple bars represent gene fusion cases of prostate cancer. *TMPRSS2*, transmembrane protease serine 2; *ERG*, v-ets erythroblastosis virus E26 oncogene homolog.

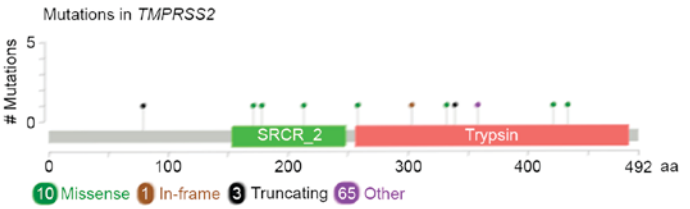
shRNA specifically reduces ERG expression in VCaP cells. To study the role of *TMPRSS2:ERG* in this context of preexisting genetic alterations, lentiviral-mediated shRNA was used to knock down *ERG* gene expression in VCaP cells

A



Cancer study	AA change		Type	Copy #	Mutation assessor	Allele freq (T)	#Mut in sample
Prostate (SU2C)	*R367C*	3D	Missense	Diploid	High	0.30	95
Prostate (broad/cornell 2012)	*P401H*	3D	Missense	Diploid	Medium	NA	43
Prostate (SU2C)	*R123C*	3D	Missense	Diploid	Low	0.37	95
NEPC (trento/cornell/ broad 2016)	*D171N*	3D	Missense	Diploid	Neutral	0.04	19

B



Cancer study	AA change		Type	Copy #	Mutation assessor	Allele freq (T)	#Mut in sample
Prostate (SU2C)	*W421C*		Missense	Diploid	High	0.07	43
Prostate (SU2C)	*D171Y*		Missense	Diploid	High	0.30	120
Prostate (FHCRC, 2016)	*G258D*	3D	Missense	AMP	Medium	0.48	1017
Prostate (FHCRC, 2016)	*G258D*	3D	Missense	Diploid	Medium	0.79	1048
Prostate (FHCRC, 2016)	*G258D*	3D	Missense	Diploid	Medium	0.48	1082
Prostate (TCGA 2015)	*I332N*		Missense	Diploid	Medium	0.05	11
Prostate (TCGA)	*I332N*		Missense	Diploid	Medium	NA	24
Prostate (SU2C)	*W213C*		Missense	Diploid	Medium	0.38	37
Prostate (TCGA)	*E178K*		Missense	DeepDel	Low	0.37	36
Prostate (SU2C)	*V433E*		Missense	Diploid	Low	0.33	37

C

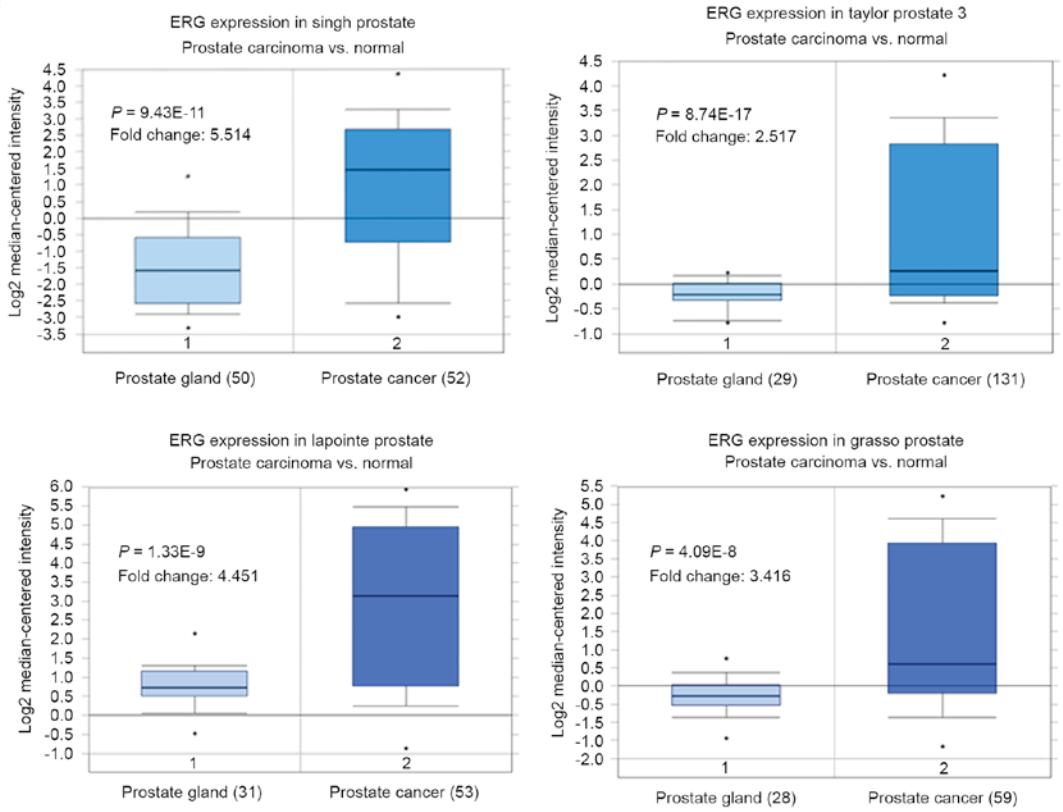


Figure 2. Missense mutations and mRNA expression of *TMPRSS2* and *ERG* in prostate cancer. Missense mutations of (A) *ERG* and (B) *TMPRSS2* in prostate cancer. (C) mRNA expression profile of *ERG* gene in four independent prostate cancer studies using Oncomine analysis. *TMPRSS2*, transmembrane protease serine 2; *ERG*, v-ets erythroblastosis virus E26 oncogene homolog.

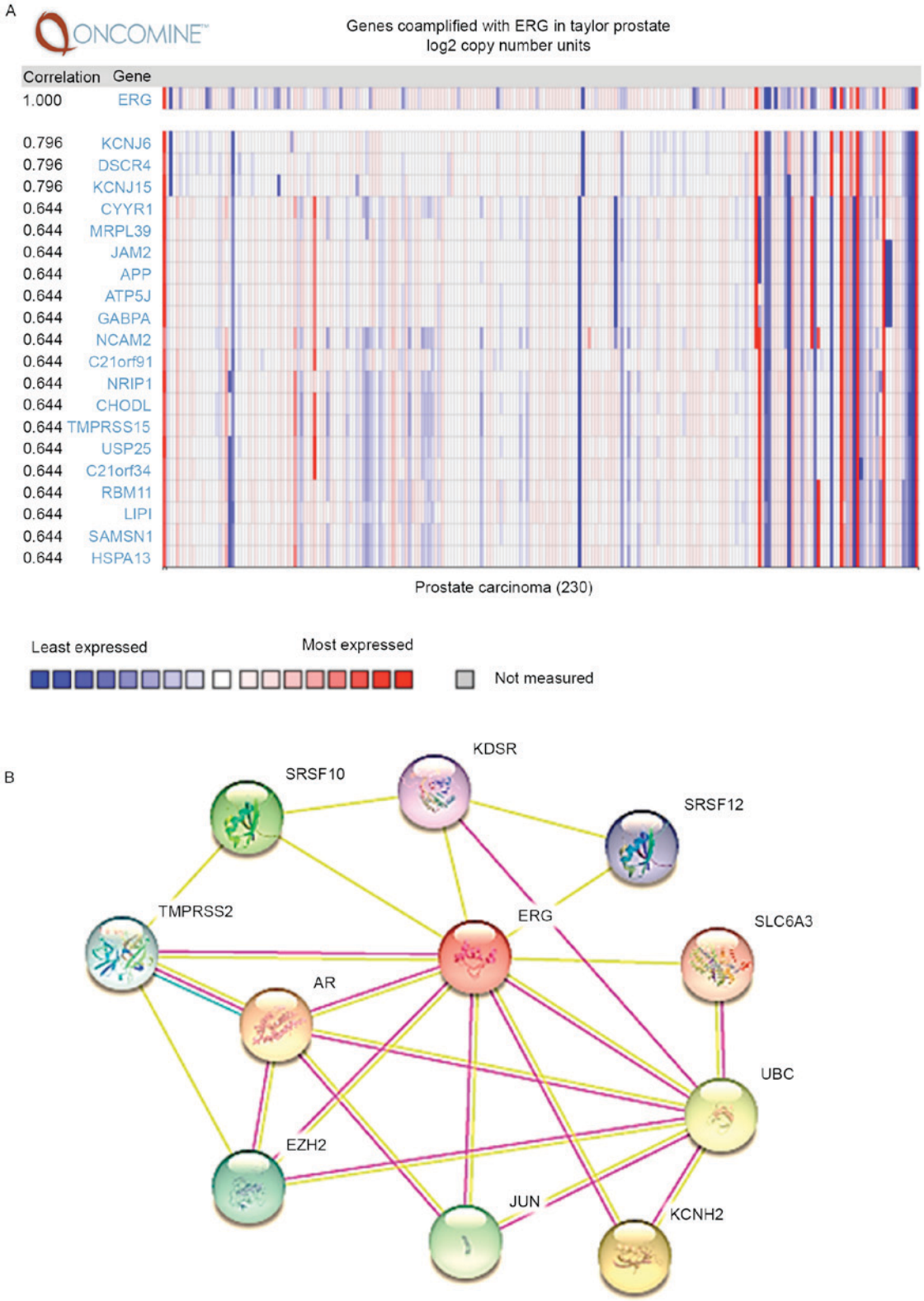


Figure 3. Co-expression and interaction networks of *ERG* in prostate cancer. (A) Co-expression analysis of *ERG* in prostate cancer using Oncomine database. (B) The interaction networks of *ERG* analyzed by STRING. *TMPRSS2*, transmembrane protease serine 2; *ERG*, v-ets erythroblastosis virus E26 oncogene homolog.

that are known to harbor the *TMPRSS2:ERG* gene fusion (11). Three pairs of shRNA of *ERG* were designed; both RT-qPCR and western blot analysis demonstrated that shRNA1-*ERG* exhibited the highest knockdown efficiency compared with the scramble control. The mRNA expression level of *ERG* was decreased by >79% and the protein expression level was reduced >93% in the shRNA-*ERG*1 viral-infected VCaP cells (Fig. 4A, $P<0.05$). In addition, there was no significant changes in cell morphology observed in the shRNA-*ERG* infected cells (Fig. 4B).

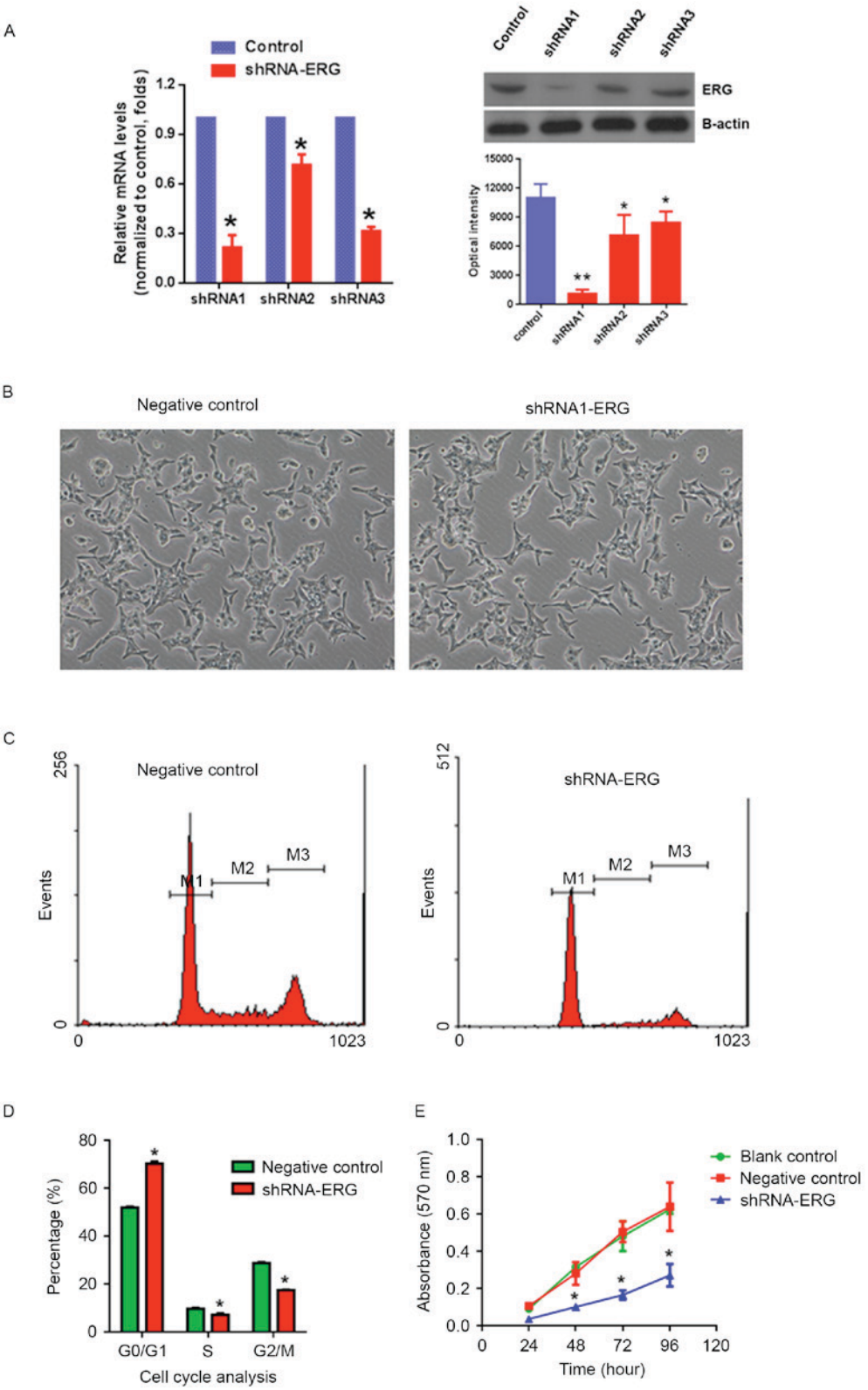


Figure 4. shRNA mediate *ERG* gene silencing and functional studies in prostate cancer cells. (A) shRNA-*ERG* knockdown efficiency studied by reverse transcription-quantitative polymerase chain reaction and western blotting. (B) Phenotype characterization of VCaP cells after *ERG* silencing by shRNA-*ERG*. (C) Flow cytometric analysis of VCAP cells and (D) % of cells in each phase of the cell cycle after *ERG* gene silencing. (E) Cell proliferation analysis using MTT methods. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. blank control or negative control. shRNA, short hairpin RNA; *TMPRSS2*, transmembrane protease serine 2; *ERG*, v-ets erythroblastosis virus E26 oncogene homolog.

Knockdown of ERG in VCaP cells inhibits cell proliferation through cell cycle arrest. Upon the shRNA-mediated knockdown of *ERG* in VCaP cells, cell proliferation was determined by MTT assay and the cell cycle distribution was

assessed by flow cytometry. The results demonstrated that specific knockdown of the *ERG* gene in prostate cancer cells could cause G_0/G_1 cell cycle arrest (Fig. 4C and D) and significantly inhibit cell proliferation (Fig. 4E) compared with the scramble virus-infected controls in VCaP cells.

Discussion

The discovery of fusion genes involving the *TMPRSS2* promoter region with *ERG* coding DNA sequences in >50% of prostate cancer cases has provide a significant insight for exploration of useful biomarkers for prostate cancer study and clinical treatment (11,27). However, the prognostic value of *TMPRESS2:ERG* gene fusion is a hotly debated topic in the current literature (13,28). The present study analyzed the *TMPRSS2* and *ERG* gene expression and alteration in multi-cancer types by using cBioPortal, and indicated that these genes were mostly altered in prostate cancer, and the most frequent alteration was gene fusion, which was consistent with previous studies (11). Notably, some missense mutations with high mutation assessor score were identified in the *TMPRSS2* and *ERG* gene, which may serve important roles in the gene fusion process and prostate cancer development.

Important studies in recent years clarified the significance of the *TMPRSS2:ERG* gene fusion in prostate cancer, and most of them indicated that the presence of the fusion gene product denotes an unfavorable outcome (7,15,29). The most direct consequence for the *TMPRSS2:ERG* gene fusion was the significant upregulation of the *ERG* gene, which is not normally expressed in prostate epithelia (30), and is likely to be involved in prostate cancer development by enhancing tumor angiogenesis (31). The high expression of *ERG* in prostate cancer is associated with advanced tumor stage, shorter survival time, high Gleason score and metastasis (12). Full-length *ERG* is a 486 amino-acid 54 kDa transcription factor, and contains an ETS DNA-binding domain and a pointed domain (32,33). Normally, *ERG* is highly expressed in the embryonic mesoderm and endothelium and serves a critical role in the formation of the vascular system and the urogenital tract, and in bone development (34-36). Aberrant expression of the *ERG* gene has a major impact on cell invasion (37) and metastasis (38), as well as the differentiation of prostate epithelium (39). The ERG gene is the first demonstration of constitutive oncogene activation in prostate cancer; however, the functional consequences and mechanisms of the *TMPRSS2:ERG* gene fusion are not fully understood. In particular, the co-expression genes and interaction networks have not been characterized.

Recently, interest in the *TMPRSS2:ERG* fusion gene in prostate cancer remains high, which is supposed to be a novel biomarker, therapy target, diagnostic and prognostic indicator in prostate cancer (7,36). Therefore, the present study also surveyed *ERG* gene expression by Oncomine analysis, based on RNA-Seq data, which demonstrated that the *ERG* gene was significantly increased in four independent prostate cancer study datasets. Based on these findings, the present study designed specific shRNA of the *ERG* gene for loss-of function study. It was demonstrated that *ERG* gene silencing could significantly inhibit prostate cancer cells proliferation, and induce G_0/G_1 cell cycle arrest in prostate cancer cells. These results suggested that not only the alteration of *TMPRSS2* and *ERG* gene could be a specific marker in prostate cancer, but also could be a potential therapy target in prostate cancer. However, the exact mechanism remains unclear; therefore, further studies are required to illustrate the signaling pathways involved in this progression.

Acknowledgements

The present study was supported by Shenzhen Science and Technology Program Basic Research Project (grant no. JCYJ20150402144905865).

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