Genetic features of TP53 mutation and its downstream FOXA1 in prostate cancer

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SUMMARY Metastasis is the most lethal form of prostate cancer, and finding new therapeutic targets remains a major clinical challenge. TP53 mutation has been identified to be involved in tumor progression and metastasis. Nevertheless, direct evidence of the role of TP53 mutation in prostate cancer metastasis and its underlying mechanism remain obscure. Herein, TP53 was found to be the most mutated gene in prostate cancer, and missense mutations were the primary mutation type based on bioinformatics data analysis. Subsequently, TP53 rs12947788 mutation site was significant in prostate cancer, and correlated with metastasis and tumor-node-metastasis (TNM) stage. Furthermore, forkhead box A1 (FOXA1), a target of TP53, was highly expressed in prostate cancer tissue, especially in TP53-mutant patients. It was also associated with patients' Gleason scores and nodal metastasis. Knockdown of FOXA1 suppressed the migration in prostate cancer cells in vitro. Our findings indicate that targeting TP53 mutation and FOXA1 might be a promising therapeutic target for prostate cancer metastasis.

Keywords TP53 mutation, FOXA1, bioinformatics, prostate cancer, metastasis

1. Introduction

Prostate cancer is the second malignant tumor worldwide and the fifth leading cause of cancer mortality in men (1). Metastasis is the most lethal form of prostate cancer, and it has a poor overall survival of only 30% at 5 years (2). Androgen deprivation therapy is the most common because prostate cancer cells are highly sensitive to the androgen pathway. However, relapse is inevitable. A previous study has revealed that 10-20% of patients with prostate cancer metastasis develop castration resistance within 5 years, which leads to rapid progression. Unfortunately, although the treatment strategies including enhanced hormonal or chemohormonal therapy are used in this setting, more organs show metastases because of the inconsistent efficacy. Meanwhile, the median survival time is approximately 14 months (range 9-30), which markedly increases the mortality burden of patients (3-5). More recently, evidence supports that targeting gene therapies holds great promise for the treatment of prostate cancer. However, sensitivity is low since therapeutic genes are lacking, limiting its clinical application. Therefore, finding new treatments for metastasis remains a major clinical challenge. Elucidating the underlying mechanisms of prostate cancer metastasis is imperative for developing novel therapeutic strategies for prostate cancer.

Deregulation of some genes are involved in prostate cancer progression from localized to metastatic disease, and control of genetic stability is frequently lost. TP53 on human chromosome 17, encoding a 53 kDa protein (also called cellular tumor antigen p53), plays a pivotal role in several tumors progression (6,7). Importantly, p53 exerts various effects through regulating downstream genes in prostate cancer metastatic cascade. Zhan Yang et al find that p53/RBM25-mediated circAMOTL1L-miR-193a-5p-protocadherin-α regulatory axis contributes to regulate epithelial to mesenchymal transition in prostate cancer metastatic progression (8). Results from Qiji Li et al reveal that wild-type p53 directly interacts with Frizzled8 (FZD8) promoter, participating in bone metastasis in prostate cancer by Wnt/β-catenin signaling (9). These results give us a hint that TP53 plays an essential in prostate cancer. In fact, TP53 is prone to
a gene mutation in approximately half of malignant tumors, such as colon, lung, liver, breast, skin, and bladder, which shows that TP53 mutation contributes to tumor initiation and malignant progression (10). Interestingly, the clinical significance of TP53 status in prostate cancer has been and continues to be a hot topic. Previous studies demonstrate that TP53 mutation frequency is about 10% in primary prostate cancer but up to 50% in metastases, which is associated with poor overall survival and progression-free survival (11). Prostate cancer patients with cT3 mutation in plasma have extremely rapid disease recurrence, and are associated with a significantly shorter metastasis-free survival (12). This drives us to explore the underlying mechanism of TP53 mutations in prostate cancer. Mutant TP53 attenuates wild-type p53 functions, developing worse clinical outcomes (13). Thus, reactivation of TP53 function represents an attractive therapeutic strategy for suppressing prostate cancer metastasis. However, only a few studies have investigated the effect of TP53 mutation on prostate cancer metastasis.

Forkhead box A1 (FOXA1, a member of the FOX family) is a well-studied pioneer factor and involved in embryonic development and disease progression (14). It is a crucial transcription factor in the occurrence and development of lung cancer and breast cancer (15). Interestingly, the role of FOXA1 in prostate cancer is still controversial. Study demonstrates that FOXA1 promotes prostate cancer angiogenesis (16). Whereas J Kim et al report that FOXA1 exhibits tumor-suppressing function and inhibits prostate cancer neuroendocrine differentiation (17). A previous study has revealed nuclear co-localization of mutant TP53 and FOXA1 in vivo, and mutant TP53 regulates FOXA1 expression directly at FOXA1 promoter, which is involved in pancreatic ductal adenocarcinoma metastasis (18). In prostate cancer, FOXA1 is a driver of onset and progression. It reprograms the androgen receptor binding to chromatin and regulates genes associated with cell cycle and epithelial to mesenchymal transition (19). Despite these previous findings, our understanding of the role of FOXA1 involvement in prostate cancer metastasis remains incomplete, and it needs further to be elucidated.

In this study, bioinformatics data analysis was employed to illuminate the role of TP53 mutation in prostate cancer metastasis. Subsequently, TP53 mutation and FOXA1 expression were detected in clinical specimens by Sanger sequencing and RT-qPCR, respectively. The relationship of TP53 mutation with FOXA1 expression was analyzed, and the associations of both with clinical characteristics in prostate cancer were also evaluated using multiple online analysis tools. FOXA1 expression were detected in prostate cancer tissues and cells. Further, the effects of FOXA1 knockdown in prostate cancer cells on migration were investigated.

2. Materials and Methods

2.1. Clinical samples

Fifty-six prostate cancer tissues were collected and embedded in paraffin in the Department of Urology, Qilu Hospital of Shandong University. Meanwhile, the paired normal adjacent tissues from ten of them were also collected. Ages ranged from 45 to 84 years old, and the median age was 68. No patients had been treated with chemotherapy or radiotherapy before surgery. Tumor-node-metastasis (TNM) staging was according to the 8th edition of the American Joint Committee on Cancer (AJCC). Data on demographic and clinicopathological parameters were also recorded, including age, history of smoking and alcohol intake, metastasis, differentiation, TNM stage, and Gleason score (Table 1). This study was approved by the Ethics Committee on Scientific Research of Shandong University Qilu Hospital (KYLL-2019-258).

2.2. DNA extraction

DNA was extracted using a paraffin-embedded tissue DNA extraction kit (Tiangen Biocatalyst Technology Co., Ltd, DP331-02) according to instructions. The concentration and purity were detected by Onedrop OD-1000+ spectrophotometer detector.

2.3. Sanger sequencing

A PCR amplification instrument was utilized to amplify the target fragment of TP53. Amplification cycle conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 10 min. The samples were purified using a Cycle Pure Kit (D6492-02, Omega Biotek, USA), sequenced with Big Dye Terminator v3.1 kit (Thermo Fisher Scientific, USA), then purified. Finally, sequencing analysis was performed by ABI 3500 gene sequencer.

2.4. RNA extraction and RT-qPCR

Total RNAs were isolated from prostate cancer tissues and cells using TRIzol reagent (Invitrogen, USA), then reversely transcribed into cDNA using PrimeScript TM RT reagent kit (Takara, Japan). Real-time quantitative PCR was assessed by SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Data were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and relative gene expression was calculated by the 2^−ΔΔCt method. Primers were shown in Table 2.

2.5. ICGC and cBioportal

Mutated genes in prostate cancer were analyzed by
2.8. TIMER database

Tumor IMMune Estimation Resource (TIMER, (https://cistrome.shinyapps.io/timer/) database provides three main analysis modules: Immune, Exploration, and Estimation (23). FOXA1 expression in pan-cancer tissues was obtained and analyzed through the TIMER database.

2.9. Cancer Cell Line Encyclopedia dataset

Cancer Cell Line Encyclopedia (CCLE) is a tumor genomics research project led by the Broad Institute. It collects and sorts out the omics data of cell lines (24). FOXA1 expression in prostate cancer cell lines was analyzed by the CCLE dataset (https://portals.broadinstitute.org/ccle/page?gene=FOXA1).

2.10. Immunohistochemistry

Paraffin-embedded prostate cancer tissues and its paired normal adjacent tissues were deparaffinized and endogenous peroxidase activity was blocked using 3% hydrogen peroxide. After antigen retrieval, tissues were incubated with primary antibody anti-FOXA1 (1:200, HUABIO, Hangzhou HuaAn Biotechnology CO., Ltd, China) at 4°C overnight, followed by the secondary
antibody (Cat: PV-9001, ZSGB-BIO, Beijing, China) at 37°C for 30 min. Staining was observed with DAB (Cat: ZLI-9019, ZSGB-BIO, Beijing, China). Meanwhile, DP260 Autostainer (Dakewe Biotech Co., Ltd., Beijing, China) was used for hematoxylin and eosin (H&E) staining, according to the manufacturer's instructions.

2.11. Prostate cancer cell lines and cell culture

Two prostate cancer cell lines (DU145 and PC3) were purchased from the Chinese Academy of Sciences Cell Bank (China). Cells were grown in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Invitrogen, USA), and cultured at 37°C in 5% CO₂.

2.12. Small interference RNA (siRNA) transfection

SiRNA targeting FOXA1 and stable negative control were designed and synthesized by Shanghai Generay Biotech Co., Ltd (Shanghai, China). Prostate cancer cells (2 × 10⁵/mL) were seeded in 6-well plates for 24 h. After 70% confluence, cells were transfected with FOXA1-siRNA (100 nmol/L) using Lipofectamine 3000 Transfection Kit (Invitrogen, USA), according to the manufacturer's instructions. Sequences were shown in Table 3.

2.13. Cell migration

Cell migration assays were conducted using transwell chambers. Prostate cancer cells were transfected with FOXA1-siRNA and suspended in 200 µL serum-free medium. Then cells were seeded into the upper chamber of 24-well plate, and the lower chamber was covered with 600 µL medium containing 10% FBS. After incubation for 24 h, cotton swabs were used to remove the cells remaining on the upper membrane. Migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Finally, cells were photographed under microscope (IX81, OLYMPUS).

2.14. Statistical analyses

Data were presented as mean ± standard deviation (SD). Student's t-test or one-way analysis of variance (ANOVA) was used for comparing differences between groups. Statistical analyses were performed using SPSS 25.0 software. The Pearson's chi-square test was utilized to evaluate statistical significance between the clinical variables and mutational profile. P < 0.05 was considered statistically significant.

3. Results

3.1. TP53 was the main mutated gene in prostate cancer

ICGC analysis showed that TP53 was the main mutated gene in prostate cancer (Figure 1A). Furthermore, calibration frequency of TP53 in 19 prostate cancer-related studies was analyzed through cBioportal database, and mutational information in TP53 was described (mutation and missense) (Figure 1B). The distribution of TP53 mutation was detected by exome sequencing, and results were illustrated on cBioportal database. It also showed that TP53 mutation mainly included missense variant, frameshift variant and stop gained, of which missense mutation was the most common (Figure 1C). The highly conserved sites of TP53 point mutation were R175H, R245H, R248H, R249H, R273H, and R282H (Figure 1D).

3.2. TP53 correlated with prostate cancer metastasis and TNM stage

Notably, TP53 expression differed among different mutation types and copy-number alterations in the cBioportal database (Figure 2A and B). Furthermore, 56 prostate cancer tissues were collected and detected by Sanger sequencing. Heterozygous mutation was found at TP53 rs12947788 site, and the rate was 71.4% (40/56). TP53 rs12947788 mutation was significantly associated with metastasis (p = 0.047) and TNM stage (p = 0.040), but not with age, history of smoking and alcohol intake, differentiation, or Gleason score (Table 1). These findings revealed that TP53 mutation might be involved in the occurrence and metastasis of prostate cancer.

3.3. TP53 mutation correlated with FOXA1

In a previous study, p53 participated pancreatic cancer metastasis by interacting FOXA1 (18). Herein, FOXA1 expression in prostate cancer tissues was shown to be differentially expressed between TP53-mutant and TP53-nonmutant patients, which was verified in the TCGA database (Figure 3B). Data

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense (5'-3')</th>
<th>Antisense (5'-3')</th>
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<tr>
<td>FOXA1#1</td>
<td>GGAUGUUAGGAACUGUGAA TT</td>
<td>UUCACAGUUCUAAACAUCC TT</td>
</tr>
<tr>
<td>FOXA1#2</td>
<td>GGACUUAAGGCAUAACGA TT</td>
<td>UUCCGAUUGCGAAUGUCC TT</td>
</tr>
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<td>FOXA1#3</td>
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<td>UUCGAACUAUGUUGCGGTT</td>
</tr>
<tr>
<td>Negative Control</td>
<td>UUCUCGAACUGUGUCAGUTT</td>
<td>ACGUGACACGUUCGGAAGATT</td>
</tr>
</tbody>
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from our clinical samples showed that TP53 mutation frequency was 71.4% (Figure 3C). A similar trend with an online database was also investigated (Figure 3D). FOXA1 expression was positively correlated with TP53 mutation, suggesting that TP53 mutation might promote prostate cancer metastasis by regulating FOXA1.

3.4. FOXA1 was up-regulated in prostate cancer and associated with metastasis

FOXA1 expression in pan-cancer tissues and cell lines was analyzed by the TIMER database and CCLE database, respectively. Results illustrated that FOXA1 in tumor tissues was higher than that in normal tissues, especially in prostate cancer (Figure 4A). As well, FOXA1 was more highly expressed in prostate cancer cells than in other cells (Figure 4B). Immunohistochemical experiments from our clinical specimen demonstrated that the level of FOXA1 protein
in prostate cancer tissues was significantly higher than in their paired normal adjacent tissues (Figure 4C).

Subsequently, FOXA1 expression in prostate cancer based on patients’ Gleason scores and nodal metastasis status was further explored by UALCAN database. FOXA1 expression in prostate cancer with different Gleason scores was higher than in the normal group (Figure 4D). FOXA1 expression was significantly up-regulated in prostate cancer tissues compared to normal adjacent tissues (Figure 4C).

**Figure 3.** TP53 mutation correlated with FOXA1 expression. (A) FOXA1 expression in prostate cancer based on TP53 mutation status through UALCAN database. (B) Gene expression in FOXA1 and TP53 mutation in prostate cancer from TCGA database. (C) TP53 mutation was detected by Sanger sequencing and frequency was calculated. (D) FOXA1 expression was detected by RT-qPCR in TP53 wildtype (WT) and TP53 mutation (MUT) prostate cancer tissues. *, P < 0.05; ****, P < 0.0001.

**Figure 4.** FOXA1 was up-regulated in prostate cancer and associated with tumor metastasis. (A) FOXA1 expression in pan-cancer tissues was assessed through TIMER database. *, P < 0.05; **, P < 0.01, ***, P < 0.001. (B) FOXA1 expression in cells from the CCLE database. (C) FOXA1 protein level in prostate cancer tissues and its paired normal adjacent tissue by immunohistochemistry (scale bar: 50 μm). (D) FOXA1 expression in prostate cancer based on patients’ Gleason scores was analyzed by UALCAN database. ****, P < 0.0001. (E) FOXA1 expression in prostate cancer based on nodal metastasis status by UALCAN database. ****, P < 0.0001. N1: 1 to 3 axillary lymph node.
regulated in prostate cancer lymph node metastases compared with the normal group (Figure 4E).

3.5. Knockdown of FOXA1 inhibited migration of prostate cancer cells in vitro

To explore the role of FOXA1 in regulating prostate cancer metastasis, knockdown of FOXA1 by siRNA was performed in prostate cancer cells (DU145 and PC3). The interference efficiency was evaluated using RT-qPCR. Results showed that FOXA1-siRNA#2 was the highest and used in the following experiments (Figure 5A). As reflected by transwell migration, transfection with FOXA1 siRNA could effectively inhibit migration of prostate cancer cells (Figure 5B), which indicated that up-regulation of FOXA1 promoted cell metastasis.

4. Discussion

Prostate cancer is the most frequently diagnosed cancer that seriously affects men's health (25). The incidence and mortality rates are closely related to the age (26). It is reported that prostate cancer often involves lymph node and/or bone sites metastasis, which causes most cancer-related deaths (27).

TP53, a tumor suppressor gene, is frequently altered in various cancers including prostate cancer (28). In this study, TP53 was confirmed to be the main gene in prostate cancer with high mutation frequency via ICGC and cBioportal databases. It has many mutation sites and types, and missense mutations are dominant. The relationship between gene mutation and tumor development is a complex biological process. TP53 mutations often occur in the central DNA-binding domain such as R249H and R273H and have oncogenic action. The interaction between mutant p53 and most regulatory molecules including p63 and microRNAs affects the stability of those molecules and the crucial molecular pathways involved in invasion and metastasis through regulating Zinc finger E-box binding homeobox 1 (ZEB1) and zinc finger protein 652 (ZNF652) (10). Different TP53 mutation types have different effects on TP53 expression, but missense mutation can make TP53 dysfunctional, while nonsense mutation may result in TP53 function loss. Data of exome sequencing from cBioportal database showed that the distribution of TP53 mutations in prostate cancer was very scattered.

As a third-generation genetic marker, a single nucleotide polymorphism (SNP) reflects the genetic differences between individuals, which have provided unique insights into the basis of cancer genetic susceptibility (29). Interestingly, men with gene mutations are at an increased risk of metastatic cancer, which has prompted further studies in the field. One study showed that TP53 rs1042522 polymorphism increased the susceptibility of malignant bone tumors (30). TP53 Arg72Pro (SNP rs1042522) was significantly associated with the risk of non-Hodgkin lymphoma (31). However, the functional link among TP53 polymorphism, causation of biological behavior and prognosis in prostate cancer remains elusive. In the present study, TP53 was found to have mutations in 19 prostate cancer-related studies through the cBioportal database. Our findings from clinical specimens using Sanger sequencing revealed that the rate of TP53 mutation was 71.4%, and heterozygous mutation site was at rs12947788.

Prostate cancer is prone to lymphatic spread to locoregional lymph nodes, bone marrow stroma predominantly in the axial skeleton, even distant visceral sites. This is the most lethal form of prostate cancer. Because the mechanism is poorly understood, there is no effective treatment for prostate cancer. Further analysis showed that TP53 mutation was significantly associated with metastasis and TNM stage. It was consistent with a previous report that TP53 mutations could enhance
early prognostication of prostate cancer progression (32). Deletion of wild-type p53 promoted prostate cancer cells metastasis to bones by regulating the C-X-C chemokine receptor type 4/ C-X-C motif chemokine 12 (CXCR4/ CXCL12) activity (33). This suggested that elucidating the downstream mechanism of TP53 mutation would help us find a promising therapeutic strategy.

FOXA1 is a pioneer transcription factor and essential for various type of tumor progression, including liver, bladder, prostate, and lung cancer (34). Several studies have shown that FOXA1 is a potential prognostic biomarker in prostate cancer (35,36) and has been implied to promote androgen-dependent prostate cancer growth (37). This suggests that FOXA1 might be a novel therapeutic strategy for prostate cancer. A previous study has revealed that targeting FOXA1-mediated transforming growth factor-beta (TGF-β) signaling can effectively suppress castration-resistant prostate cancer progression (38). Multiple pro-angiogenic factors induced by FOXA1 can promote prostate cancer angiogenesis (16). However, the mechanism of FOXA1 in regulating prostate cancer metastasis still remains unclear.

Our results demonstrated that FOXA1 expression was high in prostate cancer patients and cells, and significantly up-regulated in Gleason score and lymph node metastases. This may provide a strategy for assigning risk in combination with FOXA1 and Gleason scores. Furthermore, the level of FOXA1 in TP53-mutant patients was higher than in TP53-nonmutant patients. This funding was verified by data from our clinical specimen and UALCAN and TCGA database. Previous research has shown that GATA binding protein 3 (GATA3) mutations can disrupt localization of estrogen receptor-alpha (ER-α) and FOXA1 in breast cancer (39). In this study, TP53 mutations may lead to aberrant transcription factor localization and change in FOXA1 downstream transcriptional networks. Based on these results, we speculated that TP53 mutation and FOXA1 might functionally converge in modulating prostate cancer tumorigenesis and metastasis. However, the current study provides no evidence regarding the underlying molecular mechanism by which TP53 mutations may regulate FOXA1 in prostate cancer metastasis.

To further clarify this issue, the clinical significance of FOXA1 in normal and cancerous tissues from prostate cancer, as well as the function of FOXA1 in the regulation of tumor cell migration in vitro were investigated. Results showed that FOXA1 knockdown might inhibit prostate cancer cell migration. This may be related to some pathways, such as the repression of TGF-β signaling, androgen receptor pathway. Future research will investigate these mechanisms further.

In summary, our study illustrated that TP53 was the mutation gene with high frequency in prostate cancer and rs12947788 which were the main sites. FOXA1 was highly expressed in prostate cancer, especially in TP53-mutant patients, and was highly associated with Gleason scores and metastasis. Moreover, we confirmed that FOXA1 was significantly up-regulated in prostate cancer tissues, and knockdown of FOXA1 significantly suppressed migration in prostate cancer cells. This suggested that TP53 and FOXA1 might be promising therapeutic targets for inhibiting prostate cancer metastasis. However, the limitations of this study still exist, including its retrospective nature and relatively few patients. Future work will look to verify these results in multicenter studies with larger sample sizes.

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