IDH1R132H Promotes Malignant Transformation of Benign Prostatic Epithelium by Dysregulating MicroRNAs: Involvement of IGF1R-AKT/STAT3 Signaling Pathway

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Abstract
Risk stratification using molecular features could potentially help distinguish indolent from aggressive prostate cancer (PCa). Mutations in isocitrate dehydrogenase (IDH) acquire an abnormal enzymatic activity, resulting in the production of 2-hydroxyglutarate and alterations in cellular metabolism, histone modification, and DNA methylation. Mutant IDH1 has been identified in various human malignancies, and IDH1R132H constituted the vast majority of mutational events of IDH1. Most recent studies suggested that IDH1 mutations define a methylator subtype in PCa. However, the function of IDH1R132H in PCa development and progression is largely unknown. In this study, we showed that the prevalence of IDH1R132H in Chinese PCa patients is 0.6% (2/336). Of note, IDH1R132H-mutant PCa patients lacked other canonical genomic lesions (e.g., ERG rearrangement, PTEN deletion) that are common in most other PCa patients. The in vitro experiment suggested that IDH1R132H can promote proliferation of benign prostate epithelial cell RWPE-1 when under the situation of low cytokine. It could also promote migration capacity of RWPE-1 cells. Mechanistically, IDH1R132H was an important regulator of insulin-like growth factor 1 receptor (IGF1R) by downregulating a set of microRNAs (miR-141-3p, miR-7-5p, miR-223-3p). These microRNAs were repressed by the alteration of epigenetic modification to decrease the enrichment of active marker H3K4me3 or to increase repressive marker H3K27me3 at their promoters. Collectively, we proposed a novel model for an IDH1R132H-microRNAs-IGF1R regulatory axis, which might provide insight into the function of IDH1R132H in PCa development.

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Introduction
Prostate cancer (PCa) is the second leading malignancy in males and the fourth most common tumor type worldwide [1]. Currently, the established prognostic factors, Gleason score, pathological stage, and serum prostate-specific antigen (PSA), cannot precisely distinguish clinically aggressive PCAs from clinically indolent ones [2,3]. To meet this challenge, a better classification of the disease based on the underlying molecular features would be especially important in PCa. Several recent studies have explored the molecular basis of primary PCa and identified multiple recurrent genomic alterations, including mutations, DNA copy-number changes, rearrangements, and gene fusions [2].

Isocitrate dehydrogenases (IDHs) catalyze a redox reaction that converts isocitrate to α-ketoglutarate while reducing NADP to NADPH and liberating CO2. Mutations in IDHs have been...
identified in many human malignancies [4]. IDH1 mutations can cause alterations in cellular metabolism, histone modification, and DNA methylation [5]. Most recently, The Cancer Genome Atlas Research Network revealed a molecular taxonomy of PCa in which 74% of these tumors fell into one of seven subtypes defined by specific gene fusions (ERG, ETV1/4, and FLI1) or mutations (SPOP, FOXAI, and IDH1). Although the prevalence is low, IDH1 mutations may represent a methylator subtype in PCa. Interestingly, IDH1-mutant PCa patients seemed to possess fewer other common canonical genomic lesions in PCa [3]. To date, the exact biological role of IDH1 mutations has not been investigated in PCa so far.

Insulin-like growth factors 1 and 2 (IGFs) are proteins produced by the liver inducing cell proliferation, survival, and migration in many cell types [6]. IGFR1 is the receptor of IGFs. The dysregulated expression of IGFR1 has been described in many human malignancies [7]. IGFR1 is often overexpressed in PCa, and it associates with carcinogenesis, proliferation, and migration of PCa [8,9]. Targeting the IGF axis receptors showed promising antitumor effects in preclinical studies of PCa treatment [10].

MicroRNAs (miRNAs) are conserved small noncoding RNAs that act as posttranscriptional regulators of gene expression. Increasing evidence has shown that miRNAs play an important role in PCa progression [11]. Some studies suggested that IGFR1 can be regulated by miRNAs [12–14]. Here we show that IDH1R132H mediates the suppression of miRNAs (miR-141-3p, miR-7-5p, miR-223-3p), leading to the upregulation of IGFR1 which may promote malignant transformation of benign prostatic epithelium. This is the first time to systematically analyze the function of miRNAs in mutant IDH1 cells.

**Material and Methods**

**Patients**

A total of 336 paraffin-embedded tissues were retrieved from PCa patients with radical prostatectomy between 2001 and 2013 at Qilu Hospital of Shandong University (Jinan, China), Shandong Provincial Hospital (Jinan, China), General Hospital of Linyi (Linyi, China), and the Affiliated Hospital of Medical College Qingdao University (Qingdao, China). None of the patients received preoperative radiation therapy. A total of four tissue microarrays were constructed by incorporating two 1-mm cores from each representative tumor. The diagnosis was confirmed by three pathologists (B.H., M.Q., and J.H.). This study was approved by Institutional Review Board of Medical School of Shandong University (Jinan, China). Informed written consent was obtained from each patient.

**Immunohistochemistry (IHC)**

IHC was performed as previously described [15]. Briefly, the sections were incubated overnight with IDH1R132H MAb (DIA H09M) (Dianova; Hamburg, Germany), at a 1:400 dilution at 4°C and then evaluated blindly by two independent observers (B.H. and M.Q.). Cytoplasmic and nuclear immunostaining was scored into four grades (0, negative; 1-3, weak; 4-6, moderate; and 8-12, strong) based on its staining intensity (0, 1+, 2+, and 3+) and percentage of positive cells for each tumor. The details of other primers were according to previous study [18]. The details of other primers were listed in Table S1.

**Mutational Analysis**

IDH1R132H1 mutation was studied by polymerase chain reaction (PCR)–based sequencing. DNA was isolated from slices gained from formalin-fixed, paraffin-embedded (FFPE) PCa specimens using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to manufacturer’s instructions. DNA from cell lines was extracted with the Puregene Cell and Tissue Kit (Qiagen, Germany). IDH1 mutation was analyzed by PCR using forward primer 5'-ACCAAATGGCACCATACGA-3' and reverse primer 5'-TTCA TACCCGGTTAAGGTTG-3'. The product was sequenced by standard technique using primer 5'-CGGTCTTCAGAGAGC CATT-3' [17].

**Cell Culture and Reagents**

RWPE-1, LNCaP, VCAP, and 22RV1 cell lines were kindly provided by Professor Huiping Yuan (Department of Biochemistry, Shandong University). Cells were all maintained at 37°C with 5% CO2. LNCaP, VCAP, and 22RV1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Nonneoplastic, immortalized human prostatic epithelial RWPE-1 cells were cultured in defined Keratinocyte-SFM (Gibco, 17005-042) supplemented with recombinant epidermal growth factor (EGF) and bovine pituitary extract (BPE) (Gibco). AGI-5198 (Xcscbio, San Diego, CA) was made by dissolving the compound in DMSO to 10 mM as stock. The cells were treated with TGF-B1 (10 ng/ml, RD Biosciences, CA, USA) for 72 hours. Medium was changed every 24 hours.

**Plasmid Constructs and Lentivirus (LV)**

Human wild-type IDH1 cDNA (accession# NM_005896) and mutant IDH1 cDNA [NM_005896 (R132H)] were subcloned into GV358 vector (Genechem, Shanghai, China). Lentiviral vectors encoding mutant IDH1R132H (LV-GFP-IDH1R132H), wild-type IDH1 (LV-GFP-IDH1WT), and an empty vector control (LV-GFP-VECTOR) were synthesized by Genechem. Following lentiviral infection, 2 μg/ml of puromycin was used for selecting stable expressed cell lines.

**RNA Isolation and Quantitative Real-Time (qRT)-PCR Assay**

Total RNA was isolated with TRIzol (Invitrogen, CA) and reverse-transcribed into cDNA by using RevertAAce qPCR RT kit (TOYOBO, Japan). qRT-PCR assay was carried out by using FastStart Universal SYBR Green Master (BioRad, USA) according to manufacturer’s instructions. GAPDH was used as an internal loading control for mRNAs and miRNAs. Pre-U6 was used as an endogenous control for pre-miRNA. For detecting mature miRNA, qRT-PCR assay was performed using All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, USA). RNU48 was used as an internal control. The primers of Mirnas precursors were according to previous study [18]. Western blot analysis was performed as previously described [19]. Primary antibodies used were anti-IDH1R132H (DIA H09M, 1:800, Dianova), anti-IDH1 (#3997, 1:1000, CST), anti-IGFR1 (ab39675, 1:1000; abcam), anti-AKT (1:1000; CST), anti-pAKT (Ser473, 1:1000; CST), anti-STAT3 (1:1000, CST), anti-pSTAT3 (Tyr705, 1:1000, CST), and anti-GAPDH (1:1000, Santa Cruz). Three independent experiments were performed.
Transient Transfection

The transfections of miRNA mimic, miRNA inhibitor, siRNA, and their corresponding control (GenePharma) were carried out with Hiperfect transfection reagent (Qiagen) following the manufacturer’s instructions. The detail sequences were shown in Tables S2 and S3.

Immunofluorescence

Immunofluorescence was preformed according to the previous methods [20]. Cells grown on glass slides (NEST, China) were fixed and immunoprecipitated using the EZ-Magna ChIP assay (Millipore, Billerica, MA). The immunopositivity was tested by Ki67 and IGF1R. Antibodies used to immunoprecipitate purified chromatin were approved by the Shandong University Animal Care Committee.

miRNA Array Bioinformatics Analysis

RNA expression of RWPE-1 cells expressing IDH1R132H and VECTOR was evaluated using Agilent Human 4x44K Gene Expression Microarrays v2 (KangCheng, Shanghai, China). MiRNA target analysis was carried out by TargetScan software (http://www.targetscan.org).

Gene Set Enrichment Analysis (GSEA)

GSEA was carried out according to standard procedure. Signatures for IGF1R upregulated were derived from GSE5225 (genes with P < .05, fold change > 2) and further analyzed by GSEA in the data set of differentially expressed mRNA of IDH1R132H and VECTOR in RWPE-1 cells.

Statistics

Statistical analysis was carried out using SPSS 20.0 software. Two-sided Student’s t test was used for two groups; one-way analysis of variance is used for statistical comparisons of three or more groups. P < .05 was considered statistically significant.

Results

Mutational Analysis of IDH1R132H in PCa Patients

In addition to tumors of the central nervous system and leukemia, IDH1 mutations have been identified at low incidence rate in other solid malignancies [23]. In the current study, we first performed immunohistochemical staining of IDH1R132H on PCa patients. A total of four tissue microarrays including 336 PCa cases were constructed according to the protocols and quantification methods reported before [16,24]. In all, 15 PCa patients were immunopositive for IDH1R132H. Among them, 11 showed weak scale, and 4 were moderate or strong scale for IDH1R132H. Representative IHC images of IDH1R132H staining intensity were shown in Figure 1A. To further characterize the IDH1 gene status, IDH1R132H IHC-positive PCa samples were further examined by PCR-based sequencing. In total, 2/336 (0.6%) PCa samples were confirmed IDH1R132H (G to A) positive (Figure 1C). To avoid false-negative rate of this antibody, we also sequenced 90 IHC-negative patients for IDH1 mutations. No IDH1R132H mutation was found in these patients. Besides, the mutations of PCa cell lines RWPE-1, LNCaP, VCAP, and 22RV1 were also tested. No IDH1 mutations were found in these cell lines.
The Promotion of Malignant Transformation of Benign Prostatic Epithelium by IDH1R132H

At present, there are still controversial arguments about the functions of IDH1 mutations [25–27]. To further characterize the role of IDH1R132H in PCa development, we stably transfected the nontransformed prostate benign epithelial cell RWPE-1 and PCa cell LNCaP with lentiviral vectors encoding GFP-tagged mutant IDH1 (IDH1R132H), wild-type IDH1 (IDH1WT), or empty vector (VECTOR). The mRNA and protein expression levels of IDH1R132H were verified by qRT-PCR and Western blot (Figures 2, A and B, and S1, A and B). As shown in Figure 2C, in the absence of cytokines (1/4 normal-dose EGF and BPE), the growth rate of IDH1WT and VECTOR RWPE-1 cells was gradually sluggish, but RWPE-1 cells expressing IDH1R132H became cytokine independent and had significant growth advantage. In addition, wound-healing and Transwell assays demonstrated that IDH1R132H can promote migration of RWPE-1 cells (Figure 2, D and E). These data suggested that IDH1R132H might have the ability to induce malignant transform of benign prostate epithelial cells. We then evaluated the function of IDH1R132H in PCa cell line. However, different from benign prostatic epithelial cells, the capacities of proliferation and migration were decreased in IDH1R132H-mutant LNCaP cells (Figure S1, C and D). By contrast, interestingly, IDH1R132H-mutant LNCaP cells grew more rapidly than IDH1WT and VECTOR LNCaP cells in vivo (Figure S1, E and F). Many studies showed that mutant IDH is associated with cell differentiation and stem cell features [28,29]. Therefore, we next tentatively tested stem cell marker CD133 in IDH1R132H-expressed cells. qRT-PCR revealed that IDH1R132H could promote the expression of CD133 in both RWPE-1 and LNCaP cells (Figure S2, A and B).

AGI-5198, a specific inhibitor of mutant IDH1R132H, attenuates D-2-hydroxyglutarate production, delays growth, and promotes differentiation of glioma [30]. We next sought to determine whether AGI-5198 would similarly inhibit carcinogenesis activity in IDH1-mutant benign prostate epithelial cells. IDH1R132H-mutant RWPE-1 cells were treated with increasing concentrations (0 to 20 μM) of AGI-5198 and analyzed using wound-healing assay after 72 hours of
treatment. The result showed that 20 μM AGI-5198 significantly inhibited migration of IDH1R132H-mutant RWPE-1 cells (Figure S3). Effects of AGI-5198 on cell viability and migration capacity were assessed using MTS and Transwell assay. AGI-5198 treatment at 20 μM inhibited proliferation in the absence of cytokine and migration in IDH1R132H-expressing RWPE-1 cells (Figure 2, G and F).

Downregulation of miR-141-3p/miR-7-5p/miR-223-3p by IDH1R132H in RWPE-1 Cells

As miRNAs were increasingly recognized as important regulators of gene expression and other biological processes during the progression of PCA [11,31], we asked whether alterations in miRNA expression were associated with IDH1R132H-induced malignant transformation in RWPE-1 cells. Based on the literature review, we chose a curated set of prostate-specific 15 miRNAs [11,32–35] and analyzed the expression of miRNAs precursors in RWPE-1 cells stably expressing IDH1R132H, IDH1WT, and VECTOR (Figure 3A). We selected miR-223, miR-141, and miR-7-1, the most evidently downregulated miRNAs with characterization of tumor suppressor, for conducting further experiment. Utilizing qRT-PCR analysis, we showed that the expressions of pri-miR-223, miR-141, and miR-7-1, and mature miR-141-3p, miR-7-5p, and miR-223-3p were all significantly decreased in IDH1R132H-mutant RWPE-1 cells (Figure 3, B and C).

Figure 2. IDH1R132H promotes malignant transformation of prostate benign epithelial cell RWPE-1. (A, B) Western blot and qRT-PCR analysis of protein and mRNA expression levels of wild-type IDH1 and IDH1R132H in RWPE-1 cells, following stably expressing empty vector, wild-type, or mutant IDH1R132H. (C) IDH1R132H enhances the cytokine-independent growth of benign prostatic epithelial cells. The growth of stable RWPE-1 cells expressing IDH1R132H, IDH1WT, and VECTOR was examined by MTS assay. Cells were cultured under cytokine-poor conditions (1/4 normal dose EGF and BPE). (D, E) IDH1R132H promotes migration of benign prostatic epithelial cells. The migration of IDH1R132H on RWPE-1 cells was examined by wound-healing assay and Transwell migration assay. (F, G) The growth and migration of RWPE-1 cells. Cells were treated with 20 μM AGI-5198 or DMSO and analyzed using MTS and Transwell migration assay. All data represent mean ± SD of at least three independent replicates. *P < .05, **P < .01.
Mutations in IDH1 acquired a neomorphic activity that converts α-ketoglutarate to D-2-hydroxyglutarate, altering specific histone marks and inducing extensive DNA hypermethylation [5,28,36]. We hypothesized that the alternations of epigenetic modifications might contribute to the decrease of miRNAs, and the enrichments of active marker H3K4me3 and repressive marker H3K27me3 in promoters of the miRNAs were analyzed. Four pairs of primers (Table S1) were used for detecting possibly altered sites in each miRNA promoter (Figure 3, D-F). As shown in Figure 3, D-F, the enrichments of H3K4me3 were specifically reduced at the P3 and P4 promoter regions of miR-141; P2, P3, and P4 promoter regions of miR-223; and P2 and P4 promoter regions of miR-7-1 in IDH1R132H-mutant RWPE-1 cells. The enrichments of H3K27me3 were increased at P2 promoter of miR-223 and P2 promoter of miR-7-1 (Figure 3, D and F). Taken together, our data revealed that the alternations of histone modifications might contribute to the repression of miRNAs.

**Increased IGF1R expression by IDH1R132H-Downregulated miRNAs**

To further characterize the mechanism of miRNAs during RWPE-1 cell malignant transformation, we applied TargetScan prediction software (http://www.targetscan.org) to identify the potential targets of the miRNAs. A total of 15 genes are their common targets (Figure 4A), in which IGF1R was included. A variety of studies have elucidated that the IGF network is associated with the early stage of carcinogenesis and neoplasm growth and is a potential therapy target in PCa [8,37]. To verify whether the expression IGF1R was actually regulated by IDH1R132H, we evaluated the protein levels of IGF1R in IDH1R132H, IDH1WT, and VECTOR RWPE-1 cells. Both Western blot and immunofluorescence showed that IDH1R132H enhanced IGF1R protein expression compared with IDH1WT and VECTOR (Figure 4, B and C), and the overexpression of IGF1R was more evident under the treatment of
Figure 4. IDH1R132H-downregulated miRNAs lead to increased IGF1R expression. (A) The predicted target gene analysis of miR-141-3p, miR-223-3p, and miR-7-5p was performed by TargetScan software. (B, C) Western blot and immunofluorescence were used to examine IGF1R protein levels in RWPE-1 cells expressing IDH1R132H, IDH1WT, and VECTOR. (D) The protein levels of IGF1R were assessed in IDH1R132H-mutant RWPE-1 cells after treatment with miR-141-3p, miR-223-3p, and miR-7-5p mimics or negative control (Western blot). (E) Diagram indicates the recognition sites for miR-141-3p, miR-223-3p, and miR-7-5p in IGF1R 3′-UTR region, respectively. (F) WT- and mutated recognition sites of miR-141-3p, miR-223-3p, and miR-7-5p in IGF1R 3′-UTR region. (G) LUC reporter assay was performed in HEK293T cells. Cells were transiently co-transfected with wt- or mutated IGF1R 3′-UTR region together with corresponding miRNA mimics, respectively. After incubation for 48 hours, luciferase activities were measured. (H, I) Heat map and scatter diagram were used to indicate the different gene expression of IDH1R132H and VECTOR in RWPE-1 cells. (G) IGF1R-upregulated (GSE5225) gene signatures were further analyzed by GSEA in mRNA microarray of RWPE-1 cells expressing IDH1R132H and VECTOR. FDR \( q = 0.065 \). All histograms represent mean ± SD of at least three independent replicates. *P values: \*P < .05, **P < .01.
TGF-β (Figure S4A). To estimate whether IDH1R132H regulates IGF1R expression through inhibiting miRNAs, we screened the protein expression levels of IGF1R after inhibiting the endogenous and overexpressing exogenous miRNAs in VECTOR and IDH1R132H RWPE-1 cells. Exogenous mimics of miR-141-3p, miR-7-5p, and miR-223-3p reduced IGF1R expression in protein level in IDH1R132H-mutant RWPE-1 cells (Figure 4D). By contrast, inhibitors of the miRNAs enhanced IGF1R protein level in VECTOR RWPE-1 cells (Figure S4B). To determine whether this effect was direct, we assayed luciferase (LUC) reporter gene expression in HEK293T cells co-transfected with a pGL3-promoter vector carrying the respective wild-type or mutant IGF1R 3′UTR and corresponding mimics of miR-141-3p, miR-7-5p, and miR-223-3p. The relative LUC activity was inhibited by mimics of three miRNAs, and such effects were not observed when treated with the mutant construction of IGF1R 3′-UTR (Figure 4, E-G).

To characterize the molecular signature associated with IDH1R132H in RWPE-1 cells, we next analyzed differential gene expression in RWPE-1 cells transfected with IDH1R132H, IDH1WT, or VECTOR using the Agilent Human 4x44K Gene Expression Microarrays v2 (Figure 4H). A total of 8931 genes were differentially expressed between IDH1R132H and VECTOR in RWPE-1 cells, 4360 genes were upregulated, and 4571 genes were downregulated in IDH1R132H-mutant RWPE-1 cells (Figure 4I). In addition, GSEA indicated that the upregulated gene signatures of IGF1R (GSE5225) were significantly enriched in IDH1R132H-mutant RWPE-1 cells (Figure 4J). These results suggested that IGF1R signaling was activated in IDH1R132H-mutant RWPE-1 cells.

Figure 5. IGF1R is required for IDH1R132H-mediated malignant transformation. (A) Western blot analysis of indicated genes in RWPE-1 cells transfected with IDH1R132H, IDH1WT, or VECTOR. (B) Representative Western blot showed the protein levels of indicated genes in IDH1R132H-mutant RWPE-1 cells after knocking down IGF1R. (C, D) MTS and Transwell assays were used to determine the ability of proliferation and migration after silencing IGF1R in IDH1R132H-mutant RWPE-1 cells. (E) Schematic illustration of IDH1R132H-stimulated RWPE-1 cells’ malignant transformation. IDH1R132H reduces the expression of miR-141-3p, miR-223-3p, and miR-7-5p by altering histone modifications in their promoter region; the reduction of miRNAs eventually leads to the activation of the IGF1R-AKT/STAT3 signaling, which leads to the malignant transformation of RWPE-1 cells. Data are means of biological triplicate and mean ± SD. P values: *P < .05.
left untreated or minimally treated with a good outcome [40]. PCa genome and transcriptome characterization has identified molecular subtypes defined by essentially mutually exclusive genetic/transcriptomic events [41], including ETS gene fusions (most commonly involving ERG) and SPINK1 overexpression. Our group previously has characterized the genomic alterations of ERG rearrangement and SPINK1 overexpression in a subset of Chinese PCa patients [15,20]. In a recent attempt of molecular subtyping of PCa, Tomlins et al. suggested that gene expression profiling of 1577 PCa cases supports three underlying molecularly defined groups: m-ERG+, m-ETS+, and m-SPINK1+/triple negative [42]. In other two studies, mutant IDH1 may be defined as a special subtype in PCa [2,3]. Our current data showed that the incidence of IDH1R132H in Chinese PCa is 0.6%. In addition, IDH1R132H-mutant PCa patients possess fewer other canonical genomic lesions that are common in most other PCa. The rare incidence and clinical pathological features were consistent with previous reports [16,43].

To the best of our knowledge, it is the first time to systematically describe the role of IDH1 mutations in PCa. Recent studies about the function of mutant IDH1 in malignancies are still contradictory. Some referred to mutant IDH1 as a driver of cellular immortalization, transformation, and tumorigenesis [44,45]. Some pointed out that mutant IDH1 was associated with decreased proliferation in vitro and with good survival in vivo [27,46]. Our study demonstrated that IDH1R132H-mutant PCa patients possess fewer other canonical genomic lesions that are common in most other PCa. The rare incidence and clinical pathological features were consistent with previous reports [16,43].

In conclusion, our data suggested that DH1R132H may define a rare subtype (<1%) in Chinese PCa patients. We showed that IDH1R132H-mutant PCa patients possess fewer other canonical genomic lesions that are common in most other PCa. The rare incidence and clinical pathological features were consistent with previous reports [16,43].

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