

Proteomic Profiling Reveals HECTD4-dependent Regulation of Protein Ubiquitination and Signaling Pathways in Prostate Cancer

YASUO TAKASHIMA^{1,2}, MASAMI TANAKA², KENGO YOSHII³, TOMOHITO YAGI^{2,4},
AYA MIYAGAWA-HAYASHINO¹ and KEI TASHIRO²

¹Department of Pathology and Applied Biology, Kyoto Prefectural University of Medicine, Kyoto, Japan;

²Department of Genomic Medical Sciences, Kyoto Prefectural University of Medicine, Kyoto, Japan;

³Department of Mathematics and Statistics in Medical Sciences, Kyoto Prefectural University of Medicine, Kyoto, Japan;

⁴Faculty of Rehabilitation, Kobe International University, Kobe, Japan

Abstract

Background/Aim: Prostate cancer (PCa) is a leading malignancy in men, and understanding its molecular mechanisms is crucial for advancing therapeutic strategies. Ubiquitination, a key post-translational modification, regulates protein degradation and signaling, playing a vital role in cancer progression. This study focuses on HECTD4, a HECT-type E3 ubiquitin ligase, to identify its ubiquitination targets and understand its role in PCa.

Materials and Methods: HECTD4 knockdown was performed in LNCaP, PC-3, and DU145 PCa cell lines. A combination of semi-quantitative PCR and liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to identify proteins with altered expression and ubiquitination profiles. Gene ontology analysis, pathway analysis, and a proliferation assay were conducted to explore the biological significance of HECTD4.

Results: We identified 1,605 downregulated and 1,736 upregulated proteins upon HECTD4 knockdown. Key proteins involved in tumor suppression and cell cycle regulation, such as NUSAP1, CDK6, and MED13L, were ubiquitinated by HECTD4. Functional annotations revealed that these targets are associated with critical pathways, including phosphoinositide 3-kinase (PI3K)-AKT, Ras-mitogen-activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR), as well as immune infiltration, drug response, and survival analysis.

Conclusion: HECTD4 regulates protein stability and activation through ubiquitination, impacting cell cycle progression, tumor suppression, and immune response in PCa. These findings suggest that HECTD4 is a promising therapeutic target, with potential applications in drug development aimed at disrupting oncogenic signaling and enhancing treatment efficacy.

Keywords: HECTD4, ubiquitin-proteasome, proteomics, prostate cancer, cell type specificity.



Kei Tashiro, Department of Genomic Medical Sciences, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-8566, Japan. Tel: +81 752515346, e-mail: tashiro@koto.kpu-m.ac.jp

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Introduction

Prostate cancer (PCa) is the most-commonly diagnosed cancer in men and the second leading cause of cancer-related death worldwide (1, 2). Androgen receptor (AR) signaling promotes PCa progression, and androgen deprivation therapy (ADT) is the standard treatment (3). However, many patients eventually develop resistance, resulting in castration-resistant PCa (3). Prostate-specific antigen (PSA) is widely used as a diagnostic marker, but its specificity is limited, particularly in premalignant lesions such as prostatic intraepithelial neoplasia (4). Periostin has been proposed as a potential biomarker for early and advanced PCa stroma due to its increased expression (5). SLC15A2 is recognized as a prognostic biomarker and potential therapeutic target in PCa (6). Lysine lactylation, a lactate-derived post-translational modification, has been reported to contribute to tumor progression (7). The transition from hormone-sensitive PCa to castration-resistant PCa (CRPC), driven by resistance to androgen deprivation therapy, remains a major clinical challenge, whereas fatty acid metabolism is implicated in this process, suggesting that CRPC cells acquire the ability to synthesize fatty acids (8). However, the underlying mechanisms and reliable biomarkers remain poorly understood.

The ubiquitin–proteasome system (UPS) is the major non-lysosomal pathway for selective protein degradation in eukaryotic cells. Proteins tagged with polyubiquitin are degraded by the proteasome, allowing regulation of protein levels and removal of misfolded proteins (9–12). The UPS regulates diverse cellular processes, including cell cycle control, antigen presentation through MHC class I, and cell growth and proliferation (13, 14). MDM2 facilitates p53 degradation through ubiquitination, thereby regulating cell cycle arrest and apoptosis, and its expression shows a weak association with distant metastasis, potentially limited by small cohort size (15, 16). The F-box protein Skp2 promotes G₁/S transition by mediating ubiquitin-dependent degradation of the CDK inhibitor p27, and its induction correlates with reduced

p27 levels in PCa (17, 18). In addition, destabilization of cyclin–CDK complexes influences immune surveillance, as combined CDK4 inhibition and anti–PD-1 therapy enhance tumor suppression and survival in animal models (19). A recent study has highlighted UPS-linked prognostic gene signatures in PCa (20). However, despite extensive investigation of UPS-related molecules, biomarkers and protein degradation mechanisms associated with the UPS in PCa remain largely unexplored.

The circular RNA (circRNA) derived from the NEIL3 gene (*circNEIL3*), cyclized by EWSR1, is upregulated in glioma and correlates with malignant progression. It promotes glioma growth *in vitro* and *in vivo* by stabilizing IGF2BP3 through inhibition of HECTD4-mediated ubiquitination (21). The *circNEIL3* is packaged into exosomes by hnRNPA2B1 and transferred to tumor-associated macrophages (TAMs), where it induces immunosuppressive properties through IGF2BP3 stabilization, thereby promoting glioma progression (21). The long non-coding RNA *HEIH* is up-regulated in cholangiocarcinoma tissues and cells and promotes cell proliferation, migration, and invasion through a ceRNA network involving miR-98-5p and HECTD4 (22). Knockdown of *HEIH* attenuates these oncogenic phenotypes *in vitro* and restrains tumor growth *in vivo* (22). In a Korean case-control study, individuals carrying the A minor allele of single nucleotide polymorphism (SNP) rs11066280 in *HECTD4* exhibited a decreased risk of colorectal cancer (CRC), and a higher low-carbohydrate diet (LCD) score was also protective (23). Moreover, the effect of dietary carbohydrate restriction on CRC risk appeared to interact with HECTD4 genotype (23). In addition, a genome-wide CRISPR-inactivation screen identified HECTD4 as a previously uncharacterized E3 ubiquitin ligase that functions as a tumor and metastasis suppressor by promoting ubiquitin-dependent degradation of COX-2 and its regulatory kinase MKK7 (24). Depletion of HECTD4 leads to increased COX-2 expression, enhanced anchorage-independent proliferation, tumorigenesis, and metastasis, and suppression of COX-2 reverses these phenotypes (24). In PCa, DOT1L

is overexpressed in AR-positive PCa and drives tumor growth through a MYC enhancer (25). Inhibition of DOT1L reduces MYC and upregulates E3 ligases HECTD4 and MYCBP2, promoting AR and MYC degradation, making DOT1L a potential therapeutic target (25). Although the aforementioned results have been reported, the biological function and target proteins of HECTD4 in PCa and other cancers are not well understood.

In this study, we performed HECTD4 knockdown in PCa cell lines and conducted proteomic analysis using liquid chromatography–tandem mass spectrometry (LC-MS/MS). By integrating these data with publicly available transcriptomic and clinical datasets from patients with PCa, we aimed to identify global protein expression changes regulated by HECTD4 as well as candidate ubiquitinated target proteins. In addition, we identified prognostic models and signatures associated with HECTD4 in patients with PCa, focusing on ubiquitin–proteasome pathways and protein activation states.

Materials and Methods

Cell culture. PCa cell lines including LNCaP, PC-3, and DU145 were purchased from RIKEN Cell Bank (RIKEN BioResource Center, Tsukuba, Japan). Cells were grown in RPMI1640 medium (Nacalai Tesque Inc., Kyoto, Japan) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and penicillin-streptomycin (Thermo Fisher Scientific) in 5% CO₂ at 37°C, according to the manufacturer's instructions. For immunoprecipitation (IP) experiments, a proteasome inhibitor, MG132 (final conc. 10 µM) (Enzo Life Sciences, Inc., Farmingdale, NY, USA) was added to the culture medium 6 hours before harvesting total protein.

Cell proliferation assay. Cell proliferation was measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), as described (26). Briefly, cells were plated in 96-well plates at 1×10⁴ cells/well. The WST-8 solution (10 µl) was added to each well and incubated for 30 min in a 5% CO₂ incubator at 37°C. The absorbance (Abs) at 450 nm

was measured with SpectraMax M2e (Molecular Devices, Tokyo, Japan).

Gene knockdown. Cells were transfected with Stealth RNAi siRNA duplexes targeting *HECTD4* (s47037, Thermo Fisher Scientific) using Lipofectamine 3000 (Thermo Fisher Scientific), as previously described (26). Cells were cultured for 48 h. Stealth RNAi siRNA Negative Control, Med GC Duplex (Thermo Fisher Scientific) was used as a negative control.

RNA extraction. Total RNA was extracted from cells using Isogen II (Nippongene, Tokyo Japan), according to the manufacturer's protocol. RNA qualities, such as 28S/18S rRNA ratio ≥1.0 were verified using RNA Pico Chips and the Bioanalyzer System (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's protocol (27).

Reverse transcription–polymerase chain reaction (RT-PCR). Total RNA isolated from cells was reverse-transcribed using a SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific), and semi-quantitative RT-PCR was conducted using TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan) and the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), as previously described (27). Electrophoresis of PCR products and gel imaging were performed according to standard protocols. Primers for *HECTD4*: 5'-AAT ATT GGA AGT TTT GAG TGA GTG C-3' and 5'-TTC CTA AGA GTT ACA GGG AGG AGA T-3', and *GAPDH*: 5'- GCA CCG TCA AGG CTG AGA AC-3' and 5'- TGG TGA AGA CGC CAG TGG A-3', were used. The *GAPDH* expression was used for normalization (28). Representative images from three independent experiments were obtained, and PCR products were quantitatively analyzed using the ImageJ software (NIH, Bethesda, MD, USA) (27, 29).

Protein extraction. Total proteins were extracted as described (30). Cells were lysed in lysis buffer consisting of 100 mM Tris (pH 8.0), 4% (w/v) SDS, 20 mM NaCl, and 10% (v/v) acetonitrile (ACN), followed by homogenization

using a closed-type ultrasonic homogenizer to achieve complete protein solubilization. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and adjusted to 0.2 µg/µl with the same lysis buffer.

Immunoprecipitation (IP). IP was performed as described (27, 29). Cells were lysed in cold Pierce IP lysis Wash Buffer (400 µl) (Thermo Fisher Scientific) with a protease inhibitor cocktail (Nacalai Tesuque Inc.), MG132 (10 µM, final conc.) (Enzo Life Sciences) and PR-619 (5 µM, final conc., deubiquitinase inhibitor) (LifeSensors Inc. Malvern, PA, USA) on ice for 10 min. Lysates were clarified by centrifugation at $15,000 \times g$ for 30 min at 4°C. Supernatants were precleared with a Pierce Classic IP Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Precleared samples were incubated with anti-ubiquitin rabbit polyclonal antibody (anti-Ub ab) (5 µg) (#BML-UG9511; Enzo Life Sciences) at 4°C for 1 h with rotation. Pierce Protein A/G Agarose beads (Thermo Fisher Scientific) were subsequently added and incubated at 4°C for 1 h with rotation to capture antibody–protein complexes. Beads were washed 6 times with cold Pierce IP lysis Wash Buffer, and bound proteins were eluted with 50 mM Tris-HCl (pH 8.0). Immunoprecipitated proteins were analyzed by mass spectrometry. All steps were performed on ice or at 4°C to minimize protein degradation.

Protein digestion. Protein extracts were reduced by 20 mM tris 2-carboxyethyl phosphine at 80°C for 10 min and alkylated with 35 mM 2-iodoacetamide at room temperature for 30 min in the dark, followed by cleanup and digestion using the single-pot solid phase-enhanced sample preparation (SP3) method with minor modifications. Hydrophilic and hydrophobic Sera-Mag SpeedBead carboxylate-modified magnetic particles (Cytiva, Tokyo, Japan) were mixed at 1:1 (v/v), washed, and reconstituted in distilled water (15 µg/µl). Reconstituted beads were added to samples, followed by 99.5% ethyl alcohol (EtOH; final conc. 75% v/v) and mixed

for 5 min. After discarding supernatants, pellets were washed with 80% EtOH and 100% acetonitrile (ACN). Beads were resuspended in 50 mM Tris-HCl (pH 8.0) containing 1 µg trypsin/Lys-C Mix (Promega, Madison, WI, USA) and incubated at 37°C overnight. Digested samples were acidified with 5% trifluoroacetic acid (TFA), sonicated using Bioruptor II, desalted with STAGE tip (GL Sciences Inc., Tokyo, Japan), dried, and redissolved in 2% ACN containing 0.1% TFA. Peptide concentrations were measured using Lunatic instruments (Unchained Labs, Pleasanton, CA, USA), and samples were transferred to hydrophilic MS vials.

Data-independent acquisition (DIA)-mass spectrometry (MS)-based proteomics. Peptides were injected onto a 75 µm×20 cm PicoFrit emitter (New Objective, Inc., Littleton, MA, USA) packed with C18 core-shell particles (CAPCELL CORE MP 2.7 µm, 160 Å material (Osaka Soda Co., Ltd., Osaka, Japan) and separated at 50°C using a 1 h gradient at 100 nl/min on an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). Eluted peptides were analyzed by overlapping window DIA-MS using an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). MS1 spectra were acquired at 30,000-resolution with an AGC target of 3×10^6 , and MS2 spectra were acquired at 30,000-resolution with an AGC target of 3×10^6 , maximum injection time set to “auto”, and stepped normalized collision energies of 22, 26, and 30%. DIA window placements were optimized using Skyline v4.1. FAIMS conditions were evaluated using five CV values (–40 to –60 V) and various inner and outer temperature settings. LC-MS/MS raw files were searched against human spectral libraries generated by ProSight (Max Planck Institute of Biochemistry, Martinsried, Germany) using Scaffold DIA (Proteome Software, Portland, OR, USA). Search parameters included trypsin digestion with one missed cleavage, precursor and fragment mass tolerances of 10 ppm, and cysteine carbamidomethylation as a static modification. Protein and peptide identifications were filtered at a *q*-value <1% false discovery rate (FDR). Quantification data were

log₂-transformed, filtered for $\geq 70\%$ valid values per group, and missing values were imputed using Perseus (Max Planck Institute of Biochemistry). Differential proteins were defined as ≥ 1.25 -fold change with $p < 0.05$ (Welch's *t*-test). For validation, raw files were converted to mzML using ProteoWizard's MSconvert v3.0.19254 and analyzed with DIA-NN v1.9.1. A two-step search was performed, consisting of library-free spectral library generation followed by targeted analysis using the generated library. Search parameters included cysteine carbamidomethylation as a static modification, and protein identifications were filtered at a *q*-value $< 1\%$ FDR.

Dataset. Publicly available datasets for PCa from the German Cancer Research Center (DKFZ; Helmholtz Association, Heidelberg, Germany) and The Cancer Genome Atlas (TCGA; NCI/NIH, Bethesda, MD, USA) were used to generate differential expression plots (31). *HECTD4* expression levels were compared across tumor stages in PCa patients using the Kruskal–Wallis test. TCGA mRNA expression data were obtained from the UCSC Xena (<https://xena.ucsc.edu/>) to compare TCGA tumor samples to GTEx normal samples.

Gene set cancer analysis (GSCA). We performed analyses for functional signaling pathways and immune infiltration at mRNA expression levels on altered protein subsets following the *HECTD4*-siRNA knockdown in cells, using an integrated platform for genomic, pharmacogenomic, and immunogenomic gene set cancer analysis (<https://guolab.wchscu.cn/GSCA/#/>). Proteins with a fold change of ≥ 1.25 that were upregulated or downregulated were identified and analyzed for pathway correlations ≥ 0.3 . Specifically, upregulated proteins, downregulated proteins, and proteins identified by anti-Ub ab-IP with altered expression were examined. Immune infiltration correlations were also assessed for downregulated proteins. Additionally, drug response analyses of candidate proteins regulated by *HECTD4* were conducted using pan-cancer datasets from the Cancer Therapeutics Response Portal (CTRP) and the Genomics of Drug

Sensitivity in Cancer (GDSC). Correlation coefficients between protein expression changes and drug sensitivity scores were obtained from the GSCA platform. Drug response associations were filtered to include those with $|\text{correlation}| \geq 0.3$. Among these, associations with FDR-adjusted *q*-values < 0.01 were ranked by $|\text{correlation}|$, and the top 30 associations were reported. InfiltrationScore was defined as described (32).

Survival analysis. Survival analysis was performed to evaluate the association between protein expression changes induced by *HECTD4*-siRNA knockdown and patient outcomes, as described (30, 31). Proteins showing over a 1.25-fold increase or under a 1.25-fold decrease, including those with reduced levels identified by IP using an anti-Ub ab, were correlated with cancer cell proliferation marker candidates and clinical survival data. Cox proportional hazards regression was performed to analyze biochemical recurrence (BCR)-free survival (BCRFS) (DKFZ; $n=81$).

Statistics. Clustering of altered expression of proteins was conducted with the hierarchical method using the JMP's built-in modules (SAS Institute Inc., Cary, NC, USA) (33, 34). The data were presented as mean $\pm$ standard deviation (SD) of multiple samples and statistical analyses were performed with the JMP's built-in modules (SAS Institute Inc.) and Microsoft Excel (Microsoft Japan Co., Ltd. Tokyo, Japan) as appropriate. $p < 0.05$ and $q < 0.01$ were considered statistically significant.

Results

mRNA expression and knockdown of HECTD4 in PCa cells.

First, the mRNA expression levels and knockdown of *HECTD4* were confirmed by PCR. *HECTD4* was expressed in three PCa cell lines including LNCaP, PC-3, and DU145 (Figure 1A). Furthermore, the *HECTD4* knockdown by the siRNA was confirmed in LNCaP (0.40-fold, $p < 0.001$), PC-3 (0.42-fold, $p < 0.001$), and DU145 (0.02-fold, $p < 0.01$) (Figure 1A), followed by the LC-MS/MS experiments for

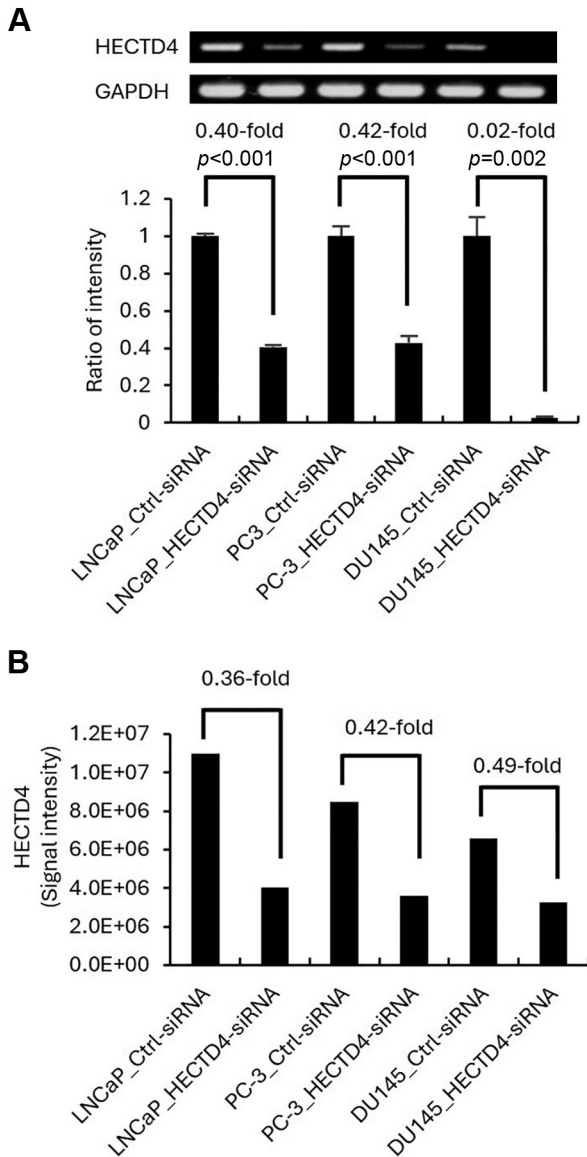


Figure 1. *HECTD4* expression and knockdown in prostate cancer cell lines LNCaP, PC-3, and DU145. (A) *HECTD4* was knocked down using siRNA, and mRNA levels were confirmed by PCR. Representative images are shown ($n=3$). GAPDH was used for normalization. (B) Decreased *HECTD4* protein was detected with LC-MS/MS ($n=1$). (Mascot; $p < 0.05$, FDR-adjusted $q < 0.01$).

protein analysis. At the protein level, *HECTD4* was largely reduced in LNCaP (0.36-fold), PC-3 (0.42-fold), and DU145 (0.49-fold) (Figure 1B). We conducted detailed analyses of how the expression of other proteins was altered by *HECTD4* knockdown as described below.

Altered expression levels of total protein and ubiquitinated protein following HECTD4 knockdown in PCa cells. Among 1,605 proteins that showed under a 1.25-fold decrease due to *HECTD4* knockdown, 36 proteins were common to all three cell types (Figure 2A). In particular, PLCH1 and TMEM53 were dramatically decreased in all three cells (fold change $< 3.0 \times 10^{-6}$) (Figure 2B). The 36 common proteins under a 1.25-fold decrease by *HECTD4*-siRNA were annotated by gene ontology terms. Representative terms included in GOTERM_BP_DIRECT were “regulation of translation” (GO:0006417; Expression Analysis Systematic Explorer score, $p=0.012$), “tRNA wobble uridine modification” (GO:0002098; $p=0.025$), “mitotic G2/M transition checkpoint” (GO:0044818; $p=0.034$), and “vesicle docking involved in exocytosis” (GO:0006904; $p=0.049$). Among 1,736 proteins that showed over a 1.25-fold increase due to *HECTD4* knockdown, 48 proteins were common to all three cell types (Figure 2C). Of these, MED13L and CCDC106 were dramatically increased in all three cells (fold change $> 8.0 \times 10^5$) (Figure 2D). Similarly, the 48 common proteins over a 1.25-fold increase by *HECTD4*-siRNA were annotated by gene ontology terms. Representative terms included in GOTERM_BP_DIRECT were “positive regulation of phosphatidylinositol 3-kinase/protein kinase B signal transduction” (GO:0051897; $p=0.007$), “post-embryonic development” (GO:0009791; $p=0.009$), “positive regulation of mesenchymal stem cell proliferation” (GO:1902462; $p=0.014$), “T cell receptor signaling pathway” (GO:0050852; $p=0.025$), “chondroitin sulfate biosynthetic process” (GO:0030206; $p=0.038$), “negative regulation of chondrocyte differentiation” (GO:0032331, $p=0.046$), and “hippo signaling” (GO:0035329; $p=0.046$). Representative terms included in GOTERM_MF_DIRECT were “N-acetylgalactosaminyl-proteoglycan 3-beta-glucuronosyltransferase activity” (GO:0050510; $p=0.008$), “glucuronosyl-N-acetylgalactosaminyl-proteoglycan 4-beta-N-acetylgalactosaminyltransferase activity” (GO:0047238; $p=0.013$), “ATP binding” (GO:0005524; $p=0.017$), “protein binding” (GO:0005515; $p=0.022$), and “acetylgalactosaminyltransferase activity” (GO:0008376).

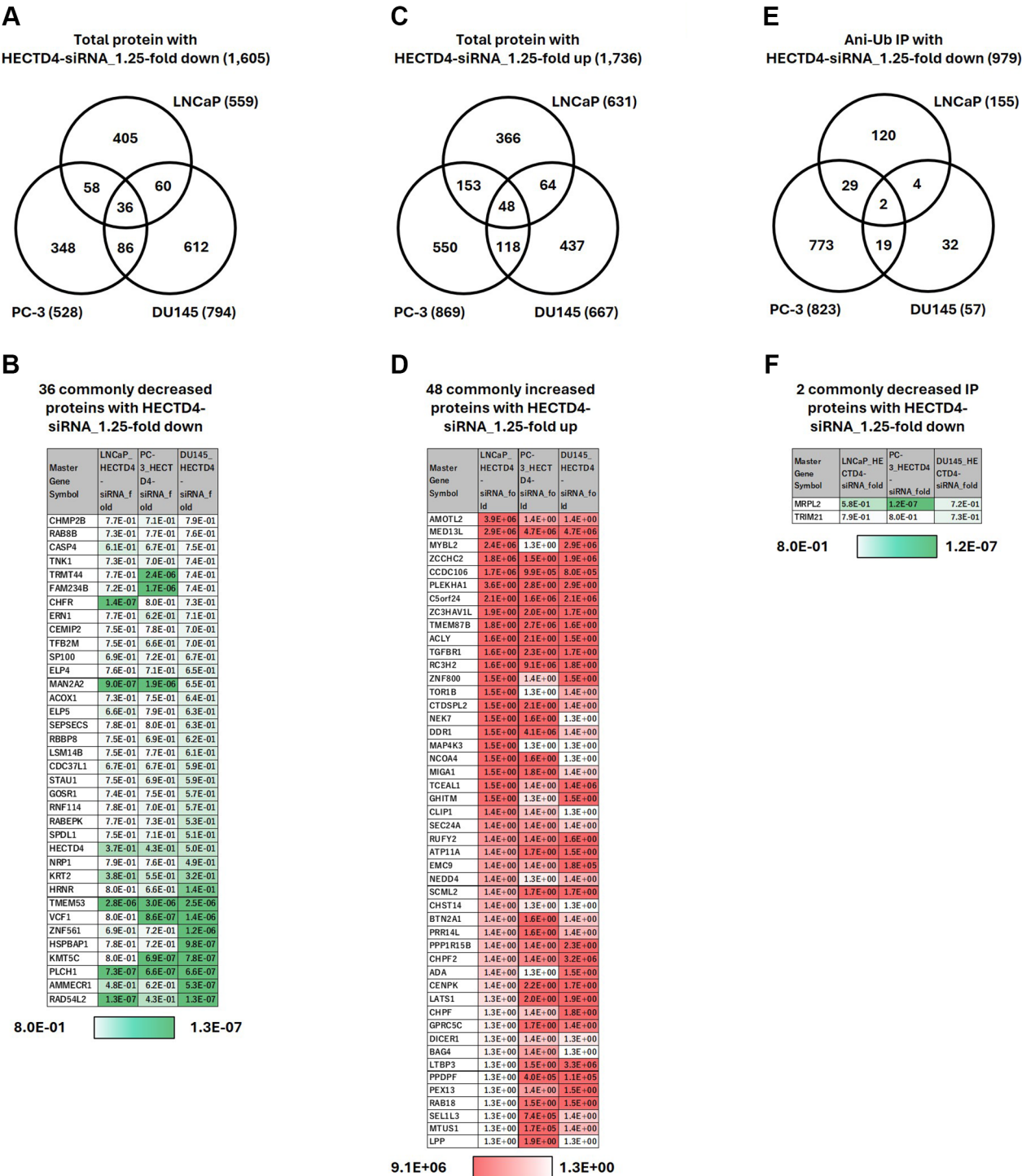


Figure 2. Summary of the altered total proteins after HECTD4 knockdown and decreased proteins in anti-ubiquitin antibody-immunoprecipitation in prostate cancer cell lines LNCaP, PC-3, and DU145. (A, B) Total proteins under a 1.25-fold decrease with the HECTD4-siRNA. The 36 commonly decreased proteins in the three cell lines. (C, D) Total proteins over a 1.25-fold increase with the HECTD4-siRNA. The 48 commonly increased proteins in the three cell lines. (E, F) The anti-Ub ab-immunoprecipitated proteins under a 1.25-fold decrease with the HECTD4-siRNA. The two commonly decreased proteins in the three cell lines.

$p=0.023$), “protein kinase activity” (GO:0004672; $p=0.040$). Among 979 ubiquitinated proteins that showed under a 1.25-fold decrease upon HECTD4 knockdown, two proteins were common to all three cell types (Figure 2E). The two proteins were MRPL2 and TRIM21 (Figure 2F). Also, the two ubiquitinated proteins MRPL2 and TRIM21 under a 1.25-fold decrease by *HECTD4*-siRNA were annotated by gene ontology terms including “ribonucleoprotein complex” ($p=0.020$) in GOTERM_CC_DIRECT and “Ribonucleoprotein” ($p=0.026$) in UP_KW_MOLECULAR_FUNCTION. These results suggest that HECTD4 regulates both protein expression and ubiquitination in PCa cells, affecting processes such as translation, cell cycle progression, signaling pathways, and ribonucleoprotein complex function.

Cell type-specific candidates for HECTD4 targets in PCa cells. Next, we attempted to identify candidate HECTD4 ubiquitination target proteins in the UPS, defined as proteins whose total levels over a 1.25-fold increase while their ubiquitinated forms under a 1.25-fold decrease upon HECTD4 knockdown. In LNCaP, PC-3, and DU145 cells, 3, 20, and 2 proteins were identified, respectively (Figure 3A, C, E). In LNCaP, candidate HECTD4 target proteins were NUSAP1, PIGH, and SOS1 (Figure 3B). These proteins were annotated with the gene ontology term in GOTERM_CC_DIRECT “cytoplasm” ($p=0.0516$). Similarly, in PC-3, there were CENPU, DCAF4, ENY2, GTSE1, LDAF1, MRPL43, MRPL49, MRPL50, NUSAP1, PPIH, RBM42, S100A8, SLC25A17, SMAD5, TARS3, TMEM41B, TYMP, UQCRCQ, WDR76, and ZNF281 (Figure 3D). These proteins were annotated with the gene ontology term in GOTERM_BP_DIRECT “mitochondrial translation” ($p=0.001$), “stem cell differentiation” ($p=0.003$), “translation” ($p=0.023$), “chemotaxis” ($p=0.040$), “mRNA splicing, via spliceosome” ($p=0.004$), “autophagy” ($p=0.016$), “positive regulation of DNA-templated transcription” ($p=0.011$), and “negative regulation of gene expression” ($p=0.018$). Additionally, identified terms were also included in GOTERM_MF_DIRECT “microtubule binding” ($p=0.002$), “protein binding” ($p=0.002$), “structural constituent of

ribosome” ($p=0.013$), and “DNA-binding transcription repressor activity, RNA polymerase II-specific” ($p=0.030$), UP_KW_DISEASE “Primary mitochondrial disease” ($p=0.001$), UP_KW_MOLECULAR_FUNCTION “Ribosomal protein” ($p=0.001$) and “Ribonucleoprotein” ($p=0.004$), and UP_KW_PTM “Acetylation” ($p=0.021$). In DU145, CDK6 and RIT1 proteins were annotated with the gene ontology term in GOTERM_MF_DIRECT “nucleotide binding” ($p=0.009$) and GOTERM_BP_DIRECT “signal transduction” ($p=0.012$) (Figure 3F). NUSAP1 was common in LNCaP and PC-3 cells (Figure 3B, D). These results suggest that HECTD4 regulates the ubiquitination of distinct sets of target proteins in a cell type-specific manner in PCa cells. Candidate targets showed increased total protein levels but decreased ubiquitination upon HECTD4 knockdown, implicating HECTD4 in processes such as translation, mitochondrial function, autophagy, and signal transduction. Notably, NUSAP1 was identified as a potential shared target in both LNCaP and PC-3 cells.

HECTD4 knockdown regulates cell signaling, cell cycle, EMT, and immune responses in PCa cells. Pathway annotation of common proteins showing over a 1.25-fold increase or under a 1.25-fold decrease with HECTD4 knockdown in three PCa cell lines was performed using transcriptome data from 500 PCa patients. For proteins over a 1.25-fold increase, ZCCH2, ZC3HAV1L, PRR14L, PPP1R15B, NEED4, and LATS1 activated phosphoinositide 3-kinase (PI3K)-AKT, Ras-mitogen-activated protein kinase (MAPK), receptor tyrosine kinase (RTK), and tuberous sclerosis complex-mammalian target of rapamycin (TSC-mTOR) signaling, while PDPF, CHST4, CCDC106, and ADA inhibited these pathways (Figure 4A). TMEM87B and SET1L3 inhibited DNA damage, epithelial-mesenchymal transition (EMT), and estrogen receptor (ER) signaling, while TCAL1, LTBP3, BTN2A1, AMOTL2, and ADA activated EMT (Figure 4A). For proteins under a 1.25-fold decrease, ZNF561, TNK1, RAD54L2, HECTD4, ERN1, and ACOX activated PI3K-AKT, Ras-MAPK, RTK, and TSC-mTOR signaling, while RABEPK and LSM14B inhibited these pathways (Figure 4B). TNK1, CHMP2B, and CDC37L1

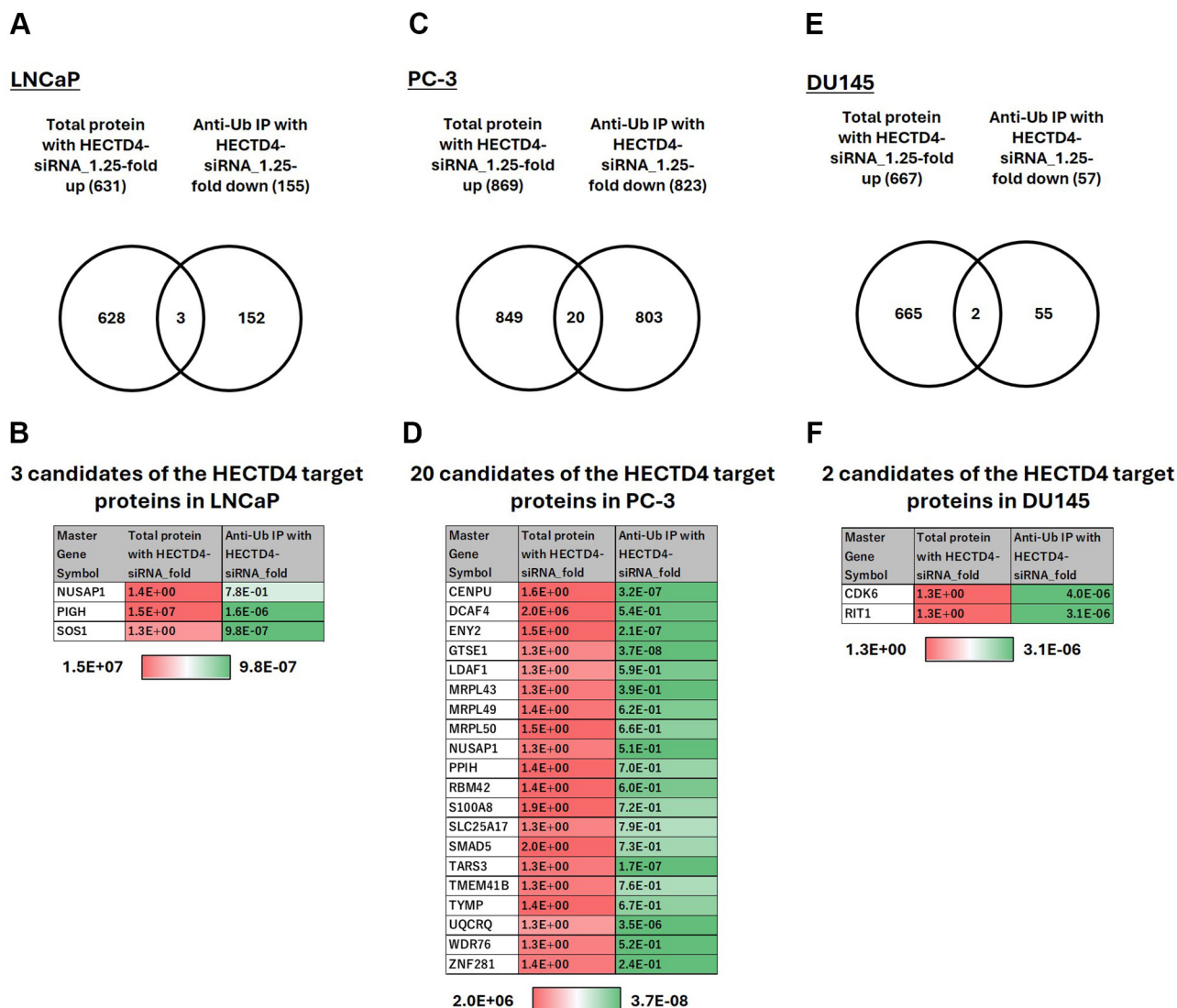


Figure 3. Candidate HECTD4 target proteins in prostate cancer cell lines LNCaP, PC-3, and DU145. (A, B) Candidate HECTD4 target proteins in LNCaP. Three proteins were detected as total proteins over a 1.25-fold increase and anti-Ub IP under a 1.25-fold decrease under HECTD4 knockdown conditions. (C, D) Candidate HECTD4 target proteins in PC-3. The 20 proteins were detected as total proteins over a 1.25-fold increase and anti-Ub IP under a 1.25-fold decrease under HECTD4 knockdown conditions. (E, F) Candidates of HECTD4 target proteins in DU145. Two proteins were detected as total proteins over a 1.25-fold increase and anti-Ub IP under a 1.25-fold decrease under HECTD4 knockdown conditions.

inhibited DNA damage, while TFB2M, SEPSECS, GOSR1, and ACOX1 inhibited EMT (Figure 4B). In HECTD4-ubiquitinated protein degradation targets, ZNF281, SOS1, S100A8, and CDK6 activated PI3K-AKT, Ras-MAPK, RTK, and TSC-mTOR signaling, while UQCRCQ, RBM42, PPIH, MRPL43, GTSE1, and ENY2 inhibited these pathways (Figure 4C). PPIH, NUSAP1, GTSE1, ENY2, and CENPU

activated cell cycle, DNA damage, and ER signaling, while S100A8 inhibited DNA damage and ER signaling (Figure 4C). For immune infiltration, SP100, ERN1, RAD54L2, PLCH1, and DASP4 activated naïve CD4, CD4 T, cytotoxic T, CD8 T, natural killer (NK) cells, and T follicular helper (Tfh) cells, while SP100, PLCH2, and CASP4 inhibited T helper type 17 (Th17) cells, monocytes, and neutrophils

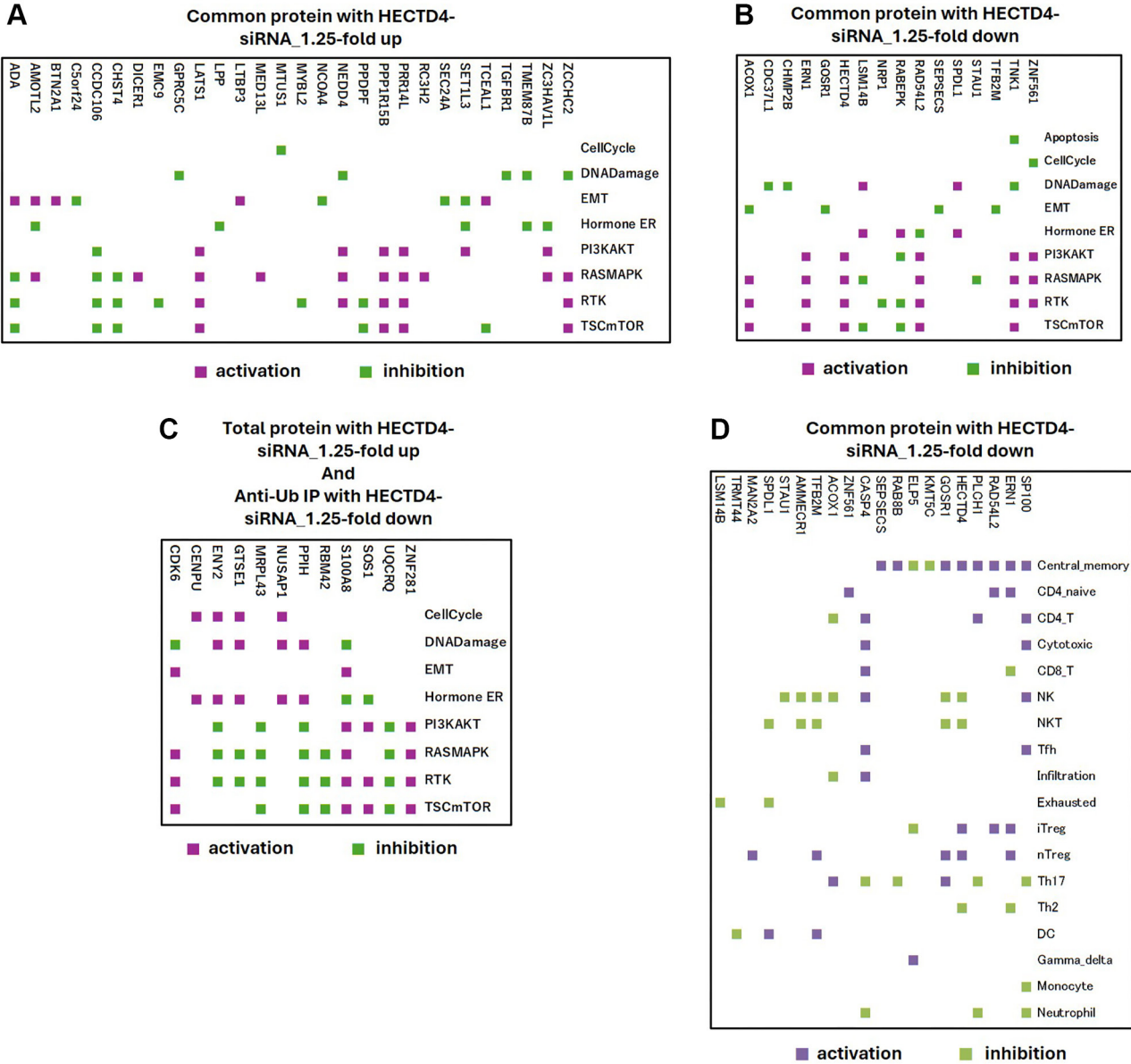


Figure 4. Signaling pathway analysis and immune infiltration analysis of the altered protein subsets with the HECTD4-siRNA knockdown in prostate cancer cell lines LNCaP, PC-3, and DU145. (A-C) Correlations of signaling pathways and the altered protein subsets with the HECTD4-siRNA knockdown in LNCaP, PC-3, and DU145 ($n=550$). (A) Common proteins over a 1.25-fold increase with HECTD4-siRNA in the three cell lines. (B) Common proteins under a 1.25-fold decrease with HECTD4-siRNA in the three cell lines. (C) Total proteins over a 1.25-fold increase and the anti-Ub IP proteins under a 1.25-fold decrease with HECTD4-siRNA. (D) Correlations between immune infiltration and the altered protein subsets with the HECTD4-siRNA knockdown in the three cell lines ($n=550$). Common proteins under a 1.25-fold decrease with HECTD4-siRNA.

(Figure 4D). HECTD4, GOSR1, TFB2M, and AMMECR1 inhibited NK and natural killer T (NKT) cells, while ERN1 and HECTD4 activated induced regulatory T (iTreg) and natural regulatory T (nTreg) cells (Figure 4D). These

results suggest that HECTD4 regulates key signaling pathways, cell cycle, EMT, and immune responses in PCa cells, impacting both tumor growth and immune modulation.

Correlation between HECTD4-regulated proteins and drug responses in PCa cells. The correlation between drug responses and proteins with HECTD4 knockdown was also investigated. Since Figure 5 summarizes correlations with an absolute value >0.3 and an FDR-adjusted q -value <0.01, the representative proteins and drugs from the most notable findings in each are described below. For the proteins that were upregulated over a 1.25-fold, SCML2, CTDSPL2, GPRC5C, and DICER1 were correlated with resistance to Tozasertib, GSK-J4, GSK461364, Lapatinib, and Teniposide, respectively (Figure 5A). For the proteins downregulated under a 1.25-fold, RAD54L correlated with susceptibility to BRD-K30748066, PX-12, and Necrosulfonamide (Figure 5B). Decreased HECTD4 itself was also correlated with susceptibility to Belinostat, PRIMA-1, and PX-12 (Figure 5B). For the HECTD4-target protein candidates, WDR76, CENPU, and PPIH were correlated with susceptibility to GSK-J4, GSK-J4, and K30740866, respectively (Figure 5C). Conversely, WDR76, RIT1, and NUSAP1 were correlated with resistance to Trametinib, GSK-J4, and Trametinib, respectively (Figure 5D). These results suggest that HECTD4-regulated proteins are associated with drug responses in PCa cells. Upregulated proteins, such as CLIP1, AMOTL2, and PPDPE, correlated with resistance to specific drugs, while downregulated proteins, including RAD54L and HECTD4 itself, were linked to increased drug sensitivity. Candidate HECTD4 targets, such as WDR76, CENPU, and PPIH, also showed correlations with both drug resistance and susceptibility, indicating that HECTD4 may influence therapeutic responses through modulation of its target proteins.

Prognostic significance of HECTD4-regulated proteins in biochemical recurrence-free survival in PCa. Furthermore, we investigated whether HECTD4-regulated proteins are associated with survival outcomes. For genes encoding proteins whose expression was altered by HECTD4 knockdown, prognostic analyses were performed to determine whether PCa patients with expression levels above the cohort median exhibited better or worse survival in BCRFS ($n=81$) (Table I). BCRFS was estimated with

the Cox proportional hazards regression model. Hazard ratio (HR) <0.8 or HR >1.25 with $p<0.05$ are shown. First, among the favorable prognosis factors, several genes exhibited significantly reduced HRs for BCRFS. Specifically, *SP100* (HR=0.496, 95% CI=0.289-0.853, $p=0.011$), *CDK6* (HR=0.581, 95% CI=0.374-0.902, $p=0.016$), *MTUS1* (HR=0.774, 95% CI=0.615-0.975, $p=0.029$), *PEX13* (HR=0.791, 95% CI=0.638-0.981, $p=0.032$), *ZNF561* (HR=0.734, 95% CI=0.552-0.976, $p=0.033$), *NEDD4* (HR=0.267, 95% CI=0.079-0.905, $p=0.034$), and *ELP4* (HR=0.641, 95% CI=0.421-0.975, $p=0.038$) were significantly associated with favorable prognosis, indicating a lower risk of BCR. In contrast, several genes were identified as poor prognostic factors, showing significantly increased HRs for BCRFS. *GTSE1* (HR=2.662, 95% CI=1.503-4.715, $p=0.001$), *KRT2* (HR=18.311, 95% CI=3.069-109.252, $p=0.001$), *DCAF4* (HR=2.893, 95% CI=1.500-5.581, $p=0.002$), *ZC3HAV1L* (HR=1.863, 95% CI=1.109-3.131, $p=0.019$), *WDR76* (HR=1.987, 95% CI=1.115-3.543, $p=0.020$), and *ADA* (HR=1.595, 95% CI=1.064-2.391, $p=0.024$) were significantly associated with poor prognosis, indicating a higher risk of BCR. These findings suggest that HECTD4-mediated protein regulation may play an important role in tumor cell progression and influence patient outcomes in PCa.

HECTD4 knockdown inhibits PCa cell proliferation and is reduced in advanced tumor stages. Finally, we performed a cell proliferation assay using the WST-8 reagent and assessed *HECTD4* gene expression in primary and metastatic stages of 92 early-onset (EO) cases from the DKFZ dataset. *HECTD4*-siRNA treatment significantly reduced cell proliferation after 3 days of culture in LNCaP (0.37-fold, $p=6.04\times10^{-7}$), PC-3 (0.38-fold, $p=4.49\times10^{-4}$), and DU145 cells (0.28-fold, $p=4.16\times10^{-4}$) (Figure 6A). In the EO PCa cases from the DKFZ cohort, *HECTD4* expression showed a decreasing trend in pT4 tumors compared with pT2 and pT3 tumors, although these differences were not statistically significant (Figure 6B). Similarly, no apparent differences in *HECTD4* expression across tumor stages were observed in the EO cases from

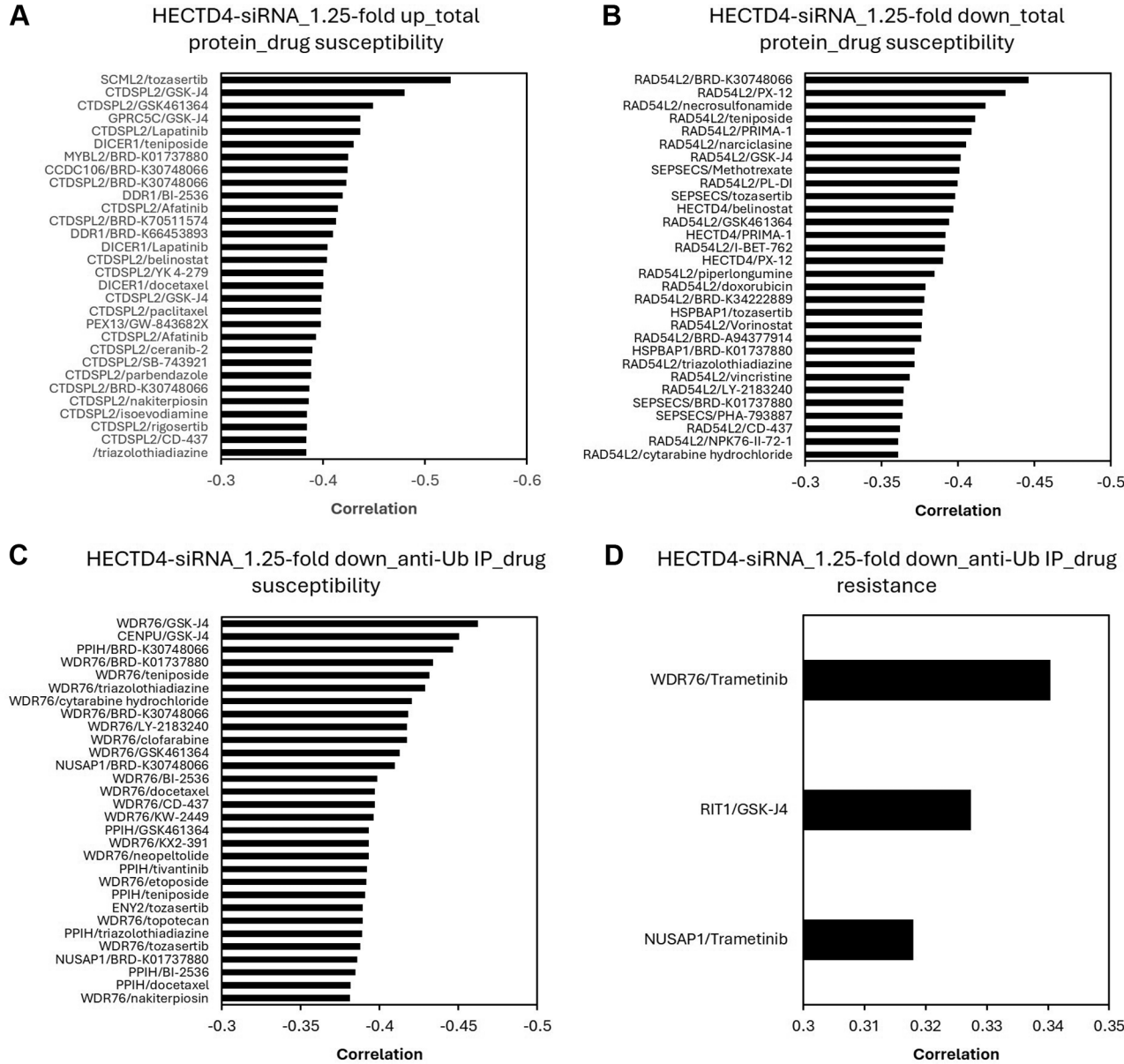


Figure 5. Drug response analysis of candidate proteins governed by HECTD4 using pan-cancer datasets from the Cancer Therapeutics Response Portal (CTRP) and Genomics of Drug Sensitivity in Cancer (GDSC). (A) Drug susceptibility to the common proteins over a 1.25-fold increase with the HECTD4 knockdown. (B) Drug susceptibility to the common proteins under a 1.25-fold decrease with the HECTD4 knockdown. (C) Drug susceptibility of the anti-ubiquitin rabbit polyclonal antibody (anti-Ub ab)-IP proteins under a 1.25-fold decrease with the HECTD4 knockdown. (D) Drug resistance of the anti-Ub ab-IP proteins under a 1.25-fold decrease with the HECTD4 knockdown. "Protein name/drug name" indicates target proteins and drugs, respectively. Drug response associations were filtered to include those with $|correlation| > 0.3$. The top 30 associations ranked by $|correlation|$ among those with FDR-adjusted q -values < 0.01 are shown.

the TCGA cohort and the total cases from the TCGA cohort (Figure 6C, D). The proliferation assay demonstrated that HECTD4 knockdown significantly suppressed cell

proliferation in multiple PCa cell lines, indicating that HECTD4 promotes PCa cell growth *in vitro*. Although the association between HECTD4 expression and tumor

Table I. Survival prediction based on highly expressed genes in prostate cancer.

	HR	95% CI	p-Value*
Favorable prognosis			
<i>SP100</i>	0.496	0.289-0.853	0.011
<i>CDK6</i>	0.581	0.374-0.902	0.016
<i>MTUS1</i>	0.774	0.615-0.975	0.029
<i>PEX13</i>	0.791	0.638-0.981	0.032
<i>ZNF561</i>	0.734	0.552-0.976	0.033
<i>NEDD4</i>	0.267	0.079-0.905	0.034
<i>ELP4</i>	0.641	0.421-0.975	0.038
Poor prognosis			
<i>GTSE1</i>	2.662	1.503-4.715	0.001
<i>KRT2</i>	18.311	3.069-109.252	0.001
<i>DCAF4</i>	2.893	1.5-5.581	0.002
<i>ZC3HAV1L</i>	1.863	1.109-3.131	0.019
<i>WDR76</i>	1.987	1.115-3.543	0.02
<i>ADA</i>	1.595	1.064-2.391	0.024

Biochemical recurrence-free survival was estimated with the Cox proportional hazards regression model. HR<0.8 and HR>1.25 with $p<0.05$ are shown. HR: Hazard ratio; CI: confidence interval. *Cox proportional hazard regression model.

stage was not statistically significant in clinical cohorts, the observed decreasing trend in advanced tumors in the DKFZ EO cohort may suggest a context-dependent role of HECTD4 during tumor progression.

Discussion

HECTD4 functions as a critical E3 ubiquitin ligase in PCa and may regulate protein stability, cell cycle progression, and multiple signaling pathways, including PI3K-AKT, Ras-MAPK, RTK, and mTOR, as indicated by this study. It also influences EMT, DNA damage responses, and the composition of immune cell infiltration, highlighting its broad role in tumor biology. Notably, NUSAP1, a microtubule-associated protein essential for spindle assembly and chromosome segregation during mitosis (35), was consistently upregulated upon HECTD4 knockdown, indicating that HECTD4 controls mitotic progression through ubiquitin-mediated degradation of NUSAP1. Dysregulation of this pathway may contribute to mitotic errors and genomic instability in cancer cells

(35). Survival analyses revealed that HECTD4-regulated genes are strongly associated with clinical outcomes. DICER1, a potential target identified by HECTD4 knockdown, suggests a possible link with microRNA regulation (36). Functionally, HECTD4 promotes PCa cell proliferation *in vitro*, yet *HECTD4* expression decreases in advanced tumors such as pT4 tumors, suggesting a context-dependent, dual role during tumor progression. Previously, using a tiling DNA microarray, we analyzed transcripts expressed in normal human prostate cells and identified a novel human gene on chromosome 12q24.13, named *POTAGE*, which consists of 26 exons and shows high homology with genes in other species (37). Since *POTAGE* is an N-terminal short splice variant of *HECTD4* expressed in normal prostate tissue, it may interact with HECTD4 at the mRNA or protein level and regulate HECTD4 activation and function in PCa.

HECTD4-regulated proteins are correlated with drug responses, with upregulated proteins often linked to resistance and downregulated proteins associated with increased sensitivity, indicating that HECTD4 and its network may serve as potential predictive biomarkers or therapeutic targets. Overall, HECTD4 coordinates a network of proteins with both tumor-suppressive and oncogenic functions, integrating cell cycle, cell signaling, EMT, and immune modulation, underscoring its potential as a key regulator and therapeutic target in PCa. The PCa cell lines used in this study are known to have distinct characteristics: LNCaP cells are AR-positive and androgen-sensitive, DU145 cells are AR-negative and serve as a castration-resistant model, and PC-3 cells are AR-negative with high invasiveness and metastatic potential (38). Among the proteins whose expression was altered by HECTD4 knockdown, a few common proteins were detected to be either upregulated or downregulated (Figure 7A). However, HECTD4 target protein candidates involved in ubiquitin-mediated protein degradation exhibited pronounced cell type specificity, suggesting that these differences may be related to the intrinsic characteristics of each cell line, such as cell type specificity or other discrepancies.

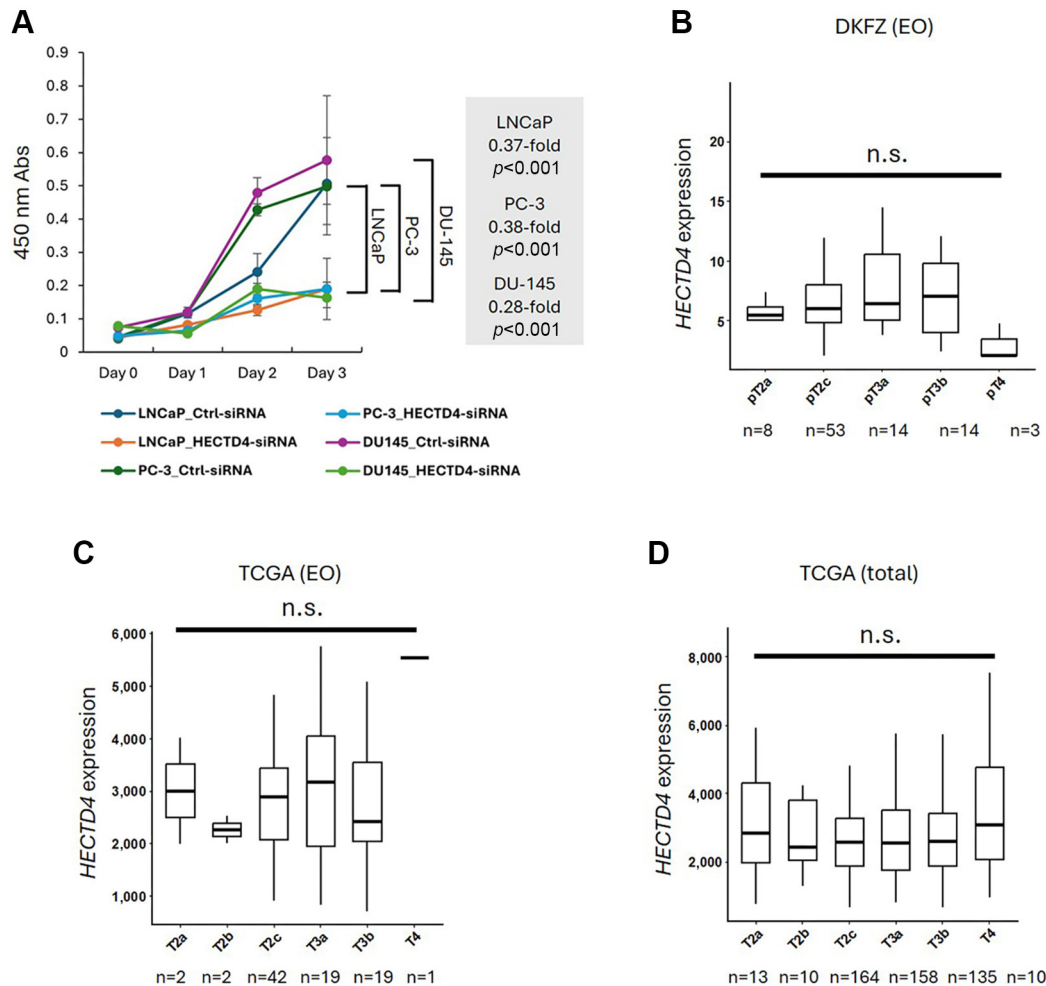


Figure 6. *HECTD4* controls cancer cell proliferation in prostate cancer cell lines LNCaP, PC-3, and DU145. (A) The *HECTD4* knockdown suppresses cell proliferation in PCa cells. Cell proliferation with *HECTD4* knockdown was examined by WST-8 assay in PCa cells. The absorbance at 450 nm was measured. Fold change and statistical significance at Day 3 were calculated. (B-D) The *HECTD4* expression across tumor stages in (B) early-onset (EO) from the DKFZ ($n=92$), (C) EO from the TCGA ($n=85$), and (D) total samples from the TCGA ($n=490$). n.s.; not significant using the Kruskal–Wallis test.

These results from the study indicate that *HECTD4* regulates parallel signaling pathways through interactions with its target proteins, and that *HECTD4* may suppress the kinase–mediator complex (CDK8-MED) by downregulating MED13L (39) and may also reduce CDK6 protein by 26S proteasome-dependent degradation after the ubiquitination of CDK6 (40) (Figure 7B). Our findings are consistent with a hypothesis that MED13L forms a kinase–mediator complex and promotes the expression of unidentified tumor suppressor genes through gene

regulatory mechanisms (Figure 7C). Furthermore, the proposed models suggest that *HECTD4* positively or negatively regulates cancer cell proliferation by inhibiting MED13L and CDK6 (Figure 7C).

On the other hand, in the favorable prognostic factors, *HECTD4* knockdown reduced SP100 and ZNF561, while CDK6 showed decreased ubiquitination and increased total protein levels. These proteins (SP100, ZNF561, and CDK6) are linked to activation of PI3K–AKT, Ras–MAPK, RTK, and mTOR signaling, as well as cell cycle

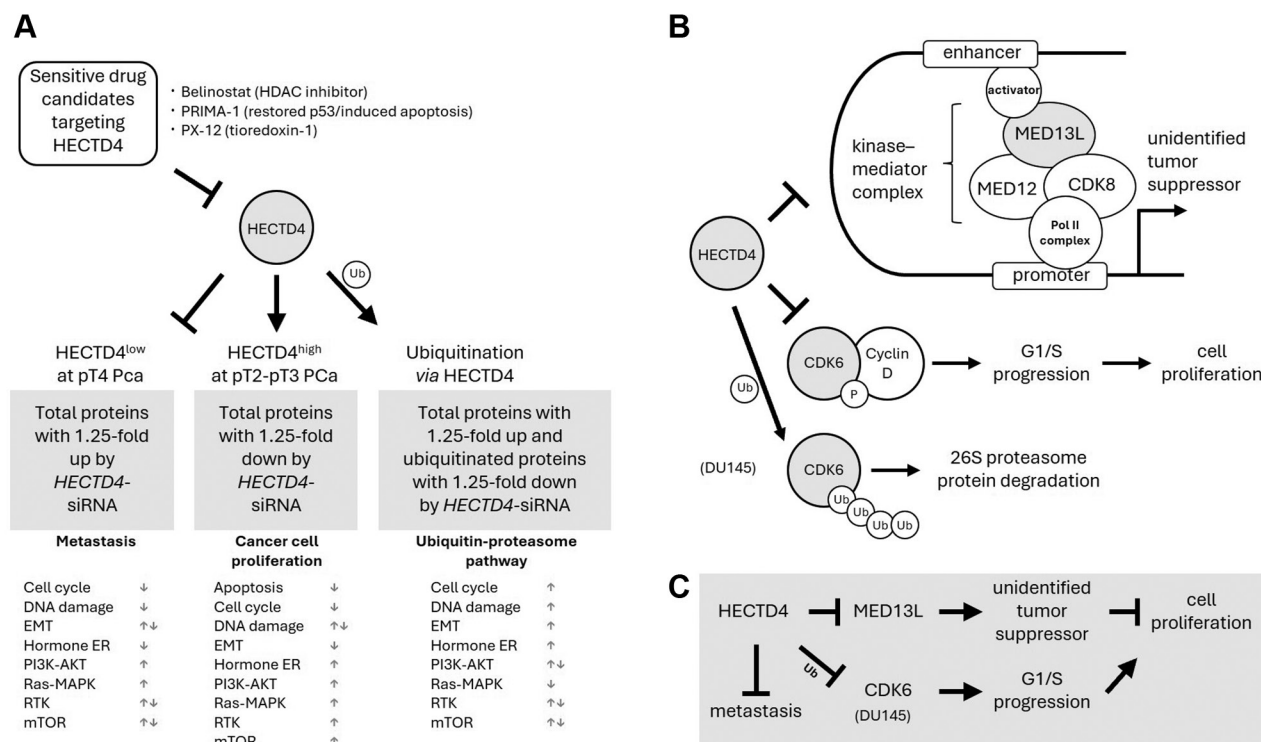


Figure 7. Proposed models of HECTD4-mediated signaling cascades in the prostate cancer cell lines LNCaP, PC-3, and DU145. (A) Parallel signaling cascades regulated by HECTD4 and target proteins, and inhibition by candidate drugs targeting HECTD4. Upper and lower arrows indicate activation and inhibition of signaling pathways, respectively. (B) HECTD4-mediated decreases in MED13L and CDK6 contribute to the maintenance of tumor cell proliferation in Pca. HECTD4 represses MED13L and CDK6, leading to reduction of the kinase-mediator complex and CDK6 degradation through the 26S proteasome pathway. MED13L is presumed to promote the expression of unidentified tumor suppressor genes through formation of the kinase-mediator complex. CDK6 is also presumed to promote G₁/S progression. (C) The proposed models suggest that HECTD4 maintains cancer cell proliferation by inhibiting MED13L and CDK6.

progression. SP100 is additionally associated with activation of CD4⁺ and CD8⁺ T cells, NK cells, and Tfh cells, suggesting a potential role in enhancing anticancer immunity. Although CDK6 promotes G₁/S progression, the association with favorable prognosis suggests a context-dependent role. This apparent discrepancy between the canonical proliferative function and clinical correlation may reflect differences between transcriptional expression and post-translational stabilization, or additional non-canonical functions of CDK6. In contrast, poor prognostic factors, including GTSE1, DCAF4, WDR76, ADA, and ZC3HAV1L, are upregulated by the HECTD4 knockdown and are involved in cell cycle regulation, DNA damage response, mitochondrial translation, EMT, and

several oncogenic signaling pathways. GTSE1, DCAF4, and WDR76 may represent therapeutic targets, and WDR76 is especially associated with GSK-J4 sensitivity and trametinib resistance. Overexpression of HECTD4 promotes context-dependent activation of Tregs and suppression of NK cells, leading to an immunosuppressive environment. Activation of other major tumor-promoting pathways, including EMT (ADA) and cell cycle/DNA damage response (GTSE1), promotes tumor growth and progression, ultimately increasing BCR risk and poor outcomes. Overall, these findings suggest that HECTD4 regulates tumor progression and immune-related pathways through ubiquitination-dependent mechanisms, highlighting its potential importance in

cancer biology and therapy. However, these potential mechanisms require further validation with *in vitro* experiments, such as HECTD4 overexpression, substrate-dependent ubiquitination assays, co-IP to assess direct-binding, and rescue experiments.

Conclusion

HECTD4 functions as a critical regulator of protein expression and ubiquitination in PCa cells, influencing translation, cell cycle, and signaling pathways. Its knockdown altered both shared and cell type-specific targets, including NUSAP1, highlighting a role in mitotic regulation and cellular homeostasis. HECTD4 also modulates key signaling pathways, including PI3K-AKT, Ras-MAPK, mTOR, EMT, DNA damage responses, and immune cell activity, linking it to tumor growth, immune modulation, and drug responsiveness. Clinically, HECTD4 supports PCa cell proliferation, yet its expression decreases in advanced tumors, suggesting a complex role in tumor progression. Survival analyses further indicate that HECTD4-regulated proteins have both protective and oncogenic effects, providing mechanistic insight into the impact on patient outcomes. Together, these findings identify HECTD4 as a multifunctional regulator in PCa, coordinating protein stability, signaling, proliferation, and therapeutic response.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Yasuo Takashima, Masami Tanaka, Kengo Yoshii, and Tomohito Yagi conducted the experiments under the supervision of Aya Miyagawa-Hayashino and Kei Tashiro. Yasuo Takashima conceived the idea for this study and wrote and edited the manuscript. All Authors read and approved the final manuscript.

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Artificial Intelligence (AI) Disclosure

During the preparation of this manuscript, a large language model (ChatGPT, OpenAI) was used solely for language editing and stylistic improvements in select paragraphs. No sections involving the generation, analysis, or interpretation of research data were produced by generative AI. All scientific content was created and verified by the authors. Furthermore, no figures or visual data were generated or modified using generative AI or machine learning-based image enhancement tools.

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