ARHGAP29 expression may be a novel prognostic factor of cell proliferation and invasion in prostate cancer

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Abstract. Yes-associated protein (YAP) is a transcription-coupling factor that plays a central role in the Hippo pathway, and its activation regulates cell proliferation and carcinogenesis. YAP activation has been reported in various malignancies, conferring tumors with migratory and invasive abilities. Several studies have suggested that YAP expression is closely associated with prostate cancer. Furthermore, YAP has been revealed to regulate destabilization of F-actin associated with the cytoskeleton via Rho GTPase-activating protein 29 (ARHGAP29), suggesting that ARHGAP29 is associated with cancer metastasis. In the present study, the functions of ARHGAP29 were examined in four prostate cancer cell lines (22Rv1, LNCaP, DU145 and PC-3) and it was revealed that upregulation of ARHGAP29 in LNCaP and DU145 cells with the lowest expression of ARHGAP29 promoted cell proliferation and invasion. Conversely, ARHGAP29 knockdown in PC-3 cells with its highest expression level significantly reduced cell proliferation and invasion. In addition, immunohistochemistry of specimens from 133 patients who underwent radical prostatectomy was performed to investigate the clinical association between ARHGAP29 expression and prognosis in prostate cancer patients. Multivariate analysis demonstrated that ARHGAP29 was an independent prognostic factor for biochemical progression-free survival (P=0.0123). These findings indicated that ARHGAP29 in prostate cancer may be a potential prognostic biomarker and therapeutic target.

Introduction

Prostate cancer was the second leading cause of cancer incidence (13.5% of approximately 9.5 million new cases) and the fifth leading cause of mortality (6.7% of approximately 5.4 million deaths) globally in 2018 for males according to Globocan (1). Advanced or metastatic prostate cancer patients usually respond well to initial androgen deprivation therapy (ADT). However, ADT does not prevent progression of prostate cancer despite the maintenance of low levels of testosterone over an extended period of time. Disease at this stage is termed castration-resistant prostate cancer (CRPC). Several systemic agents have been approved for the treatment of CRPC. However, despite the significant development of treatment options, CRPC remains as a lethal disease (2).

Genomic aberrations are common in prostate cancer cells. Various oncogenes and tumor suppressor genes are related to prostate cancer (3-8). PTEN, a tumor suppressor gene (9,10), regulates androgen receptor (AR) signaling (11) in prostate cancer. A change in AR signaling is associated with the acquisition of castration resistance in prostate cancer (12). However, the mechanism of prostate cancer progression is still not completely understood.

Since the 1990s, the Hippo signaling pathway has been revealed as a tumor suppressor signaling pathway. Yes-associated protein (YAP) plays a central role in the Hippo pathway and has been revealed to regulate cell proliferation, migration, and invasion in various cancers including prostate cancer (13). High expression of YAP has been revealed to be associated with the differentiation and extra-prostatic extension of prostate cancer (14,15). YAP expression has also been revealed to be associated with castration-resistant growth of prostate cancer cells as well as proliferation of

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Key words: Yes-associated protein, ARHGAP29, prostate cancer, LNCaP, PC-3
androgen-independent human prostate cancer cells (13,16). It is also known that YAP is activated by mechano-transduction via the hardness of the ECM (17).

YAP was revealed to bind to certain Rho GTPase-activating proteins (ARHGAPs), resulting in cytoskeletal rearrangement and the promotion of cell migration by altering the dynamics of F-actin/G-actin turnover in gastric cancer (18,19). Furthermore, some ARHGAPs are regarded as effectors of YAP (17,18). Thus far, the function of Rho GTPase-activating protein 29 (ARHGAP29) has been unclear in prostate cancer. Therefore, YAP and ARHGAP29 were examined, to investigate the role of ARHGAP29 in prostate cancer.

The aim of this study was to elucidate the role of ARHGAP29 by *in vitro* analysis and determine whether its protein expression is associated with prostate cancer prognosis.

Materials and methods

**Patients.** In total, 133 patients who underwent radical prostatectomy at Yamaguchi University Hospital from November 2000 to September 2016 were enrolled in the present study. All patients were diagnosed pathologically with prostate cancer. Detailed patient characteristics are presented in Table I. The present study was approved by the Institutional Ethics Committee of the Graduate School of Medicine of Yamaguchi University and written informed consent was obtained from all individuals enrolled in the study.

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded tissue specimens were subjected to H&E staining and immunohistochemical (IHC) staining. For each sample, 3-µm-thick sections were deparaffinized in xylene, dehydrated in ethanol, and incubated in a 0.3% hydrogen peroxide solution in methanol for 10 min at room temperature. The sections were then microwaved in a 0.01 M citrate-buffered solution (pH 6.0) for 15 min and covered in blocking solution (IMMUNO SHOT; Cosmo Bio Co., Ltd.) for 30 min at room temperature. Then, a primary antibody [anti-ARHGAP29 (1:200 dilution; cat. no. HPA026534; Atlas Antibodies) or anti-YAP (1:200 dilution; product no. 14074; Cell Signaling Technology, Inc.)] was incubated according to the manufacturers’ instructions overnight at 4˚C, followed by incubation with the respective secondary antibody (N-Histofine Simple Stain MAX PO MULTI; cat. no. 414152F; Nichirei Biosciences, Inc.) for 30 min at room temperature. To evaluate IHC staining, the H-score was used in the present study. Briefly, >500 tumor cells were counted in five different fields of vision in each section (x100, magnification), and the H-score was calculated by multiplying the percentage of positive cells by the intensity (strongly stained, 3x; moderately stained, 2x; weakly stained, 1x), yielding a possible range of 0-300 (20-22). Two independent examiners (KS and HM) judged the scores and the mean score was set to the representative score. Cut-off of the H-score was determined by receiver operating characteristic (ROC) curve.

**Cell lines.** Four primary prostate cancer cell lines (22Rv1, ATCC no. CRL-2505; LNCaP, ATCC no. CRL-1740; DU145, ATCC no. HTB-81; PC-3, ATCC no. CRL-1435) were purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 and DMEM (Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Biological Industries) and maintained in humidified incubators with 5% CO2 at 37˚C.

**siRNA knockdown of ARHGAP29.** si-ARHGAP29 and control siRNAs were obtained from Life Technologies; Thermo Fisher Scientific, Inc. siRNA sequences were as follows: ARHGAP29-#1 siRNA sense, 5'-GCAUAGGGUGUUGUGUUGUCATT-3' and antisense, 5'-UCAUAUAAACACACCUAUGCta-3'; ARHGAP29-#2 siRNA sense, 5'-GACCAAGGGCUA ACGGAAUtt-3' and antisense, 5'-AUUCGUUUAAGGCCUUGGUctc-3'; ARHGAP29-#3 siRNA sense, 5'-GCAUAGGGUGUUGUGUUGU AUCatt-3' and antisense, 5'-UCAUAUAAACACACCUAUGCta-3'. The PC-3 cell line was transiently transfected with siRNA using Lipofectamine RNAi Max (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After transfection, cells were incubated at 37˚C in a CO2 incubator for 48 h. Quantitative evaluations of mRNA and protein expression were performed by western blotting and RT-qPCR, respectively.

**Plasmid construction and transfection.** A mammalian expression of HA tagged ARHGAP29 (#104154) was purchased from Addgene, Inc. Cells were seeded on culture dishes at density of 1x10^4/well in a 6-well plate, and the pcDNA3.1 empty vector plasmid (mock) (Thermo Fisher Scientific, Inc.) or 2 µg of ARHGAP29 expressing plasmid were transfected using X-tremeGENE HP DNA transfection Reagent (Sigma-Aldrich; Merck KGaA) for 48 h, according to the manufacturer's instructions.

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<tr>
<th>Characteristics</th>
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</tr>
<tr>
<td>Initial PSA, median (range)</td>
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<tr>
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<td>98 (75)</td>
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PSA, prostate specific antigen; ADT, androgen deprivation therapy.

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**Table I. Characteristics of 133 patients who underwent radical prostatectomy.**
Regarding the DU145 cell line, plasmid transfection was performed via electroporation system using an Amaxa cell line Nucleofector Kit L (cat. no. VACA-1005; Lonza Group, Ltd.) according to the manufacturer’s instructions. Prior to electroporation, 1x10⁶ DU145 cells were centrifuged at 90 x g for 5 min, resuspended in 100 µl of Nucleofector solution and mixed with 2 µg of pmaxGFP or 2 µg of ARHGAP29 plasmid. The aforementioned cells were transferred to cuvettes and immediately electroporated based on the DU145 program (Nucleofector Program A-023) using Nucleofector 2b Device. After electroporation, cells were incubated in the cuvette at room temperature for 10 min and then 500 µl of pre-warmed RPMI-1640 medium supplemented with 10% FBS were added to the cuvette. Cells were transferred to a 6-well plate and incubated at 37°C 5% CO₂ overnight. The day after electroporation, cells were centrifuged and the medium was replaced by RPMI-1640 supplemented with 10% FBS and incubated for 48 h and then performed subsequent experiments were performed.

Reverse transcription quantitative PCR (RT-qPCR). We created cDNA by reverse transcription of mRNAs extracted from each prostate cancer cell line (22Rv1, LNCaP, DU145 and PC-3), using iScript Advanced cDNA Synthesis Kit for RT-qPCR (cat. no. 1725037; Bio-Rad Laboratories, Inc.). Quantitative real-time RT-PCR was performed in triplicate with an Applied Biosystems StepOnePlus using TaqMan universal PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The TaqMan probes and primers were purchased from Applied Biosystems. Human GAPDH (assay ID: 02786624) was used as an endogenous control. Levels of ARHGAP29 (assay ID: 00191351) and MMP-2 (assay ID: 01548727) RNA expression were determined using StepOnePlus software (version 2.2.2; Applied Biosystems; Thermo Fisher Scientific, Inc.). The miRNA expression levels were determined using the 2^(-ΔΔCt) method (23). The cycling conditions consisted of an initial denaturation at 95°C for 30 sec and PCR at 40 cycles at 95°C for 5 sec and 60°C for 30 sec.

Gene expression analysis by qPCR. Total RNA was isolated from cells (PC-3-si-NC and si-ARHGAP29), and an RT²Profiler PCR Array (Qiagen RT² Profiler PCR Array Human Cell Motility; cat. no. PAHS-128Z, product no. 330231) was used to examine the expression patterns of genes involved in human cell motility, according to the manufacturer’s instructions. We analyzed the gene expression levels and produced a heatmap using the web-based software ‘RT2 Profiler PCR Array’ Data Analysis version 3.5 (Qiagen, Inc.).

Western blotting. Cells samples were lysed in RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) supplemented with 1% protease inhibitors (completem™; Mini, cat. no. 04693124001; Sigma-Aldrich) for total protein extraction. We quantified the concentration of total proteins using BCA. Each lysate sample (30 µg protein) was separated by 4-20% SDS-PAGE (Mini-PROTEAN TGX Stain-Free Gels, cat. no. 4568095; Bio-Rad Laboratories), and then electro-transferred to a PVDF membrane. After blocking in 5% dry non-fat milk or 5% BSA for 1 h at room temperature, the membranes were incubated with a primary antibody overnight at 4°C. After washing in TBS with 0.05% Tween-20 (TBST), the membranes were incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. After washing with TBST, signals were detected using an ECL detection system (Chemidoc™ XRS+; Bio-Rad Laboratories, Inc.). Primary antibodies were as follows: anti-ARHGAP29 (product code ab85853, 1:2,000 dilution), anti-AR (product code ab133273, 1:1,000 dilution), anti-F-actin (product code ab205, 1:500 dilution) and anti-MMP-2 (product code ab97779, 1:1,000 dilution) from Abcam and anti-YAP (cat. no. 14074S; 1:1,000 dilution), anti-phosphorylated YAP (cat. no. 13008S; 1:1,000 dilution), anti-GAPDH (cat. no. 5174S; 1:1,000 dilution), anti-Cofilin (cat. no. 5175T; 1:1,000 dilution) and anti-phospho-Cofilin (cat. no. 3313T; 1:1,000 dilution) were obtained from Cell Signaling Technology Inc. Secondary antibodies were as follows: goat anti-rabbit IgG H&L (HRP) (product code ab6721; 1:10,000 dilution) and goat anti-mouse IgG H&L (HRP) (product code ab6789; 1:10,000 dilution) from Abcam. GAPDH was used for protein normalization. We performed densitometry using the public domain free software ImageJ (version 1.51; National Institutes of Health).

Cell viability and invasion assays. Cell viability was assessed using an MTS assay (CellTititer 96 AQueous One Solution Cell Proliferation Assay; Promega Corporation). After the cells were seeded at density of 5x10³/well in a 96-well plate, cell viability was measured at 24, 48, and 72 h at an OD of 490 nm. Data are expressed as the mean ± SD of three independent experiments. Cell invasion assays were performed using a CytoSelect 24-well cell invasion assay kit (Cell BioLabs, Inc.). The CytoSelect™ Cell Invasion Assay Kit contains polycarbonate membrane inserts (8-µm pore size) in a 24-well plate. The upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution. This basement membrane layer serves as a barrier to discriminate invasive cells from non-invasive cells. A cell suspension containing 0.5-1.0x10⁶ cells/ml was placed in upper chamber in serum-free media. A total of 500 µl of media containing 10% fetal bovine serum was added to the lower well of the invasion plate. After 48 h of incubation at 37°C with 5% CO₂, cells invaded through the basement membrane layer and clung to the bottom of the insert membrane. Non-invasive cells remained in the upper chamber. After removal of non-invasive cells, invasive cells were stained for 10 min at room temperature using Cell Stain Solution (Part no. 11002; CytoSelect 24-well cell invasion assay kit) and then quantified. Each insert was transferred to an empty well, 200 µl of Extraction Solution (Part no. 11003; CytoSelect 24-well cell invasion assay kit) was added per well and then incubation followed for 10 min on an orbital shaker. Subsequently 100 µl from each sample was transferred to a 96-well microtiter plate and the OD 560 nm of each sample was measured on a plate reader, according to the manufacturer’s instructions.

Database. The Cancer Genome Atlas (TCGA) accessed from the data portal of the National Cancer Institute Home Page (http://cancergenome.nih.gov/) was used for comparison with our data.

Statistical analysis. Categorical variables were compared by the Chi-squared test. Continuous variables were analyzed
using the unpaired Student's t-test when comparing two groups. One-way ANOVA followed by Tukey-Kramer test were used when comparing more than two groups. Survival analysis was estimated by the Kaplan-Meier method and compared by the log-rank test. A Cox proportional hazards regression model was used in the multivariable analysis to identify risk factors for disease progression. Statistical analysis was performed using JMP software (Pro.13; SAS Institute). P-values were two-sided, and statistical significance was defined as P<0.05 in all tests. Regarding protein expression, bivariate analysis was performed and a ROC curve was constructed using JMP software to set the cutoff value and determine the high/low expression of proteins (24).

**Results**

**AR, YAP, ARHGAP29, and F-actin expression in prostate cancer cell lines.** RT-qPCR and western blotting were performed to clarify whether there was a difference in the expression of ARHGAP29 between prostate cancer cell lines depending on AR and YAP expression. AR was expressed in 22Rv1 and LNCaP cell lines. YAP was expressed in all four prostate cancer cell lines (Fig. 1A and B). The expression level of YAP was higher in DU-145 and PC-3 cells than in LNCaP and 22Rv1 cells. ARHGAP29 protein expression was higher in PC-3 cells than in the other cell lines. F-actin was the most weakly expressed in PC-3 cells compared with the other cell lines (Fig. 1C).

**Effect of downregulation or upregulation of ARHGAP29 in prostate cancer cell lines (PC-3, LNCaP and DU145).** After downregulation (PC-3 cells) or upregulation (LNCaP and DU145 cells) of ARHGAP29 in prostate cancer cell lines (Fig. 2A and B), the expression of several proteins was examined. Based on a recent study (18), the RhoA-LIMK-cofilin signaling pathway has been revealed to be affected by ARHGAP29 in a gastric cancer cell line. Therefore, certain related genes (cofilin, p-cofilin and F-actin) were analyzed by western blotting (Fig. 2B).

After almost complete knockdown of ARHGAP in PC-3 cells, phosphorylated cofilin and F-actin were increased and cofilin expression was unchanged. In contrast, after overexpression of ARHGAP29 in DU145 cells, F-actin was slightly decreased but phosphorylated cofilin was not altered. YAP and phosphorylated YAP were slightly recovered without significant differences in si-ARHGAP29 PC-3 transfectants compared with the si-NC control. Conversely, YAP was decreased after overexpression of ARHGAP29 in DU145 cells. Expression of these proteins relative to that of the housekeeping gene GAPDH and the ratio of phosphorylated protein

Figure 1. AR, YAP, ARHGAP29, and F-actin expression in prostate cancer cell lines. (A) mRNA levels of ARHGAP29 in prostate cancer cell lines (22Rv1, LNCaP, DU145 and PC-3). Experiments were performed in triplicate. The vertical axis of the graph is presented on a logarithmic scale. The results are expressed as the mean ± SD. *P<0.01 compared with PC-3 cells. (B) Western blotting of the expression of various proteins in prostate cancer cell lines. (C) Densitometric analysis of B (relative protein expression to GAPDH). There was an inverse association between ARHGAP29 and F-actin expression, but no clear association between the expression of ARHGAP29 and that of other proteins. The results are expressed as the mean ± SD (at least three independent experiments). *P<0.05, **P<0.01 compared with PC-3 cells. AR, androgen receptor; YAP, yes-associated protein; ARHGAP29, Rho GTPase-activating protein 29.
to non-phosphorylated protein (YAP and cofilin) are presented in Fig. 2C and D.

Functional analyses by MTS and cell invasion assays were also performed in these three cell lines (Fig. 3). Cell viability and invasion were significantly decreased after downregulation of ARHGAP29 in PC-3 cells (Fig. 3A and B). After upregulation of ARHGAP29 in LNCaP and DU145 cells, cell viability and invasion were significantly increased (Fig. 3A and B).
Identification of cell motility-related genes after knock down of ARHGAP29. Based on the functional analyses, ARHGAP29 may be involved in cell proliferation or invasion. To determine new therapeutic targets or genes related to ARHGAP29 in prostate cancer cells, Qiagen RT² Profiler PCR Array Human Cell Motility was used.

The pre-designed array included 84 genes related to cell motility (Fig. 4A). Data analysis was performed using the web-based software ‘RT2 Profiler PCR Array’ Data Analysis version 3.5 as aforementioned. A heatmap is presented in Fig. 4B. When the boundary was set at 3, one gene (STAT3) was upregulated and numerous genes (including CSF1, ACTN3 and HGF) were downregulated after knocking down ARHGAP29 in PC3 cells (Fig. 4C). Regulation of some proteins, such as HGF, RHO, CAPN1, was validated by western blotting. However, there was no difference in the expression of these proteins between si-NC and si-ARHGAP29 cells (data not shown). Among the downregulated genes of the 84 genes in the array, active MMP2 expression was significantly decreased at mRNA and protein levels after knockdown of ARHGAP29 in PC3 cells (Figs. 2B and C and 4D).

Association between the expression level of ARHGAP29 and prognosis in prostate cancer patients. The expression level of ARHGAP29 was evaluated by IHC in 133 prostate cancer patients who had undergone radical prostatectomy. Representative images of YAP and ARHGAP29 staining in prostate cancer specimens (negative and positive) are presented Fig. 5A.

YAP expression was high in the nucleus of basal cells and the cytoplasm of luminal cells, but ARHGAP29 expression was high in the cytoplasm of both cells. Notably, YAP expression was unrelated to the Gleason score. The characteristics of the prostate cancer patients are presented in Table I. ARHGAP29 expression was significantly associated with the risk classification of prostate cancer (Fig. 5B). Both YAP and ARHGAP29 had low area under the curve (AUC) scores as prognostic markers, but there was a significant difference between the expression of these proteins and biochemical progression-free survival (b-PFS: P=0.0422, and P=0.0123, respectively) (Fig. 5C and D). In addition, high expression of both proteins was significantly associated with poor prognoses (Fig. 5D). In TCGA database, YAP did not exhibit a tendency for a poor prognosis in patients with high expression. In contrast, ARHGAP29 exhibited a tendency for a poor prognosis in patients with high expression. In contrast, ARHGAP29 exhibited a tendency for a poor prognosis in patients with high expression in TCGA (Fig. S1A and B). Moreover, the prognostic significance of clinicopathological parameters, including prostate specific antigen (PSA), the D’Amico risk classification, Gleason score, and pathological T category, and the expression levels of YAP and ARHGAP29 were evaluated in prostate cancer patients (Table II). As a result, high ARHGAP29 expression was a significant independent risk factor related to b-PFS in multivariate analysis (HR=2.27; P<0.05; data not shown).

Discussion

YAP has been revealed as an oncogenic protein in several cancers, such as gastric, breast, hepatocellular, pancreatic, and
Figure 4. Identification of other cell motility-related genes after knockdown of ARHGAP29. (A) The 84 genes related to cell motility are presented and analysis of the results was performed using the web-based software ‘RT2 Profiler PCR Array’ Data Analysis version 3.5. All of genes involved in the array are presented. (B) A heatmap is presented. Numerous genes were downregulated after knockdown of ARHGAP29 in PC3 cells. A1-G12 corresponds to A1-G12 of A. In the heatmap the data was displayed in a grid where each row represents a gene included in the commercial array. The color and intensity of the boxes represent changes of gene expression. For example, red represents upregulated genes and blue represents downregulated genes. (C) PCR Array of cell motility genes in PC-3 cells before and after downregulation of ARHGAP29. The graph shows the relative log value between cell motility gene expression in PC-3 cells before and after downregulation of ARHGAP29. The diagonal line in the center of the graph shows equal level of gene expression in PC-3 cells before and after downregulation of ARHGAP29. The upper diagonal lines indicate that the level of gene expression after downregulation was three times higher than that of before downregulation. The lower diagonal line indicates that the level of gene expression after downregulation was one third times lower than that of before downregulation. The mRNA level of MMP-2 was significantly downregulated. (D) Quantitative comparison of MMP-2 by RT-qPCR between si-NC and si-ARHGAP29 PC-3 transfectants. MMP-2 mRNA expression was suppressed after downregulation of ARHGAP29. Experiments were performed in triplicate. The results represent the mean ± SD. *P<0.01 compared with si-NC. ARHGAP29, Rho GTPase-activating protein 29.

Table II. Univariate and multivariate analyses of prognostic factors associated with biochemical recurrence-free survival of prostate cancer patients.

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<td>Initial PSA* (ng/ml)</td>
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<tr>
<td>&lt;20 vs. ≥20</td>
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HR, hazard ratio; CI, confidence interval; PSA, prostate specific antigen; YAP, yes-associated protein; ARHGAP29, Rho GTPase-activating protein 29.
lung cancers as well as melanoma (25-31). Similar to other cancers, YAP regulates cell migration and invasion in prostate cancer (13). Several ARHGAPs, which enhance Rho GTPase activity in almost all basic cellular processes, are oncogenic or tumor suppressor proteins (32). For example, ARHGAP5 and ARHGAP42 have been revealed to be oncogenic proteins in nasopharyngeal cancer (33,34), whereas ARHGAP24 has been demonstrated to be a tumor suppressor protein in lung, breast, and colorectal cancers (35-38). Numerous studies have demonstrated a close association of ARHGAPs with several malignancies.

Recently, other ARHGAPs such as ARHGAP18 and ARHGAP29 (17,18) were identified as transcriptional targets of YAP, and ARHGAP29 was reported as a prognostic marker for gastric cancer. Since there have been no studies on ARHGAP29 in prostate cancer, in the present study, ARHGAP29 was examined to investigate how it affects progression or metastasis of prostate cancer and whether ARHGAP29 may be a prognostic marker for prostate cancer. Initially, protein expression in four prostate cancer cell lines (22Rv1, LNCaP, DU145 and PC-3) was assessed. Among these cell lines, PC-3 and DU145 did not express AR, but highly expressed YAP. In contrast, YAP expression was low in AR-expressing cell lines (22Rv1 and LNCaP). PC-3 cells highly expressed ARHGAP29 compared with the other three cell lines. AR-null PC-3 cells are derived from bone metastasis (39,40). After complete knockdown of ARHGAP29 in PC-3 cells, their proliferation and invasion were significantly decreased. In contrast, cell proliferation and invasion were increased after upregulation of ARHGAP29 in LNCaP and DU145 cells. In the present study, we did not investigate a direct interaction between AR and ARHGAP29. However, the present results indicated that ARHGAP29 regulates cell proliferation and invasion in prostate cancer cells. Recently, Qiao et al demonstrated that ARHGAP29 suppressed the RhoA-cofilin pathway and destabilized F-actin, which caused cytoskeletal rearrangement and promoted migration (18). In the present study, certain proteins in the RhoA-cofilin pathway were analyzed in PC-3, LNCaP and DU145 cells. Specifically, phosphorylated cofilin and F-actin were recovered when ARHGAP29 was completely knocked down in PC-3 cells. Moreover, the relative protein level of phosphorylated cofilin to cofilin was increased. These data were consistent with the results from a recent study on a gastric cancer cell line (18). F-actin was slightly decreased in DU145 cells following upregulation of ARHGAP29. In PC-3 cells, ARHGAP29 may be associated with cell migration by...
suppressing the RhoA-cofilin pathway similar to a previous study (18). However, cofilin and p-cofilin expression were not altered in LNCaP and DU145 cells. Specifically, the results of LNCaP and Du145 cells were not demonstrated as the reverse of the observations made in PC-3 ARHGAP29-knockdown cells. These results may be explained by the fact that each cell line has a different genotype/phenotype as revealed in a previous study (for instance only PC-3 cells do not express α-catenin) (40). Upregulation of ARHGAP29 may lead to decrease of F-actin via another pathway in LNCaP and DU145 cells, however, to demonstrate this, further experiments are required.

Apart from the Rho-A-cofilin pathway, to identify new targets or cancer pathways related to ARHGAP29, a pre-designed array (Human Cell Motility), based on the functional analysis data in the present study, was used. Among the 84 genes in the array, expression of several genes was altered after knocking down ARHGAP29 in PC-3 cells. Among the genes, expression of MMP-2 was validated by RT-qPCR and western blotting. Among the matrix metalloproteinase (MMP) family, which degrade the ECM, MMP-2, also known as gelatinase A, is reported to be correlated with the invasion and metastasis of cancer cells as well as angiogenesis in numerous human cancer tissues (41,42). Moreover, Zhang et al indicated a role of YAP in gastric cancer and revealed that LATS1 inhibited the growth and metastasis of gastric cancer cells by restraining nuclear transfer of YAP and downregulating MMP-2 expression concurrently (43). This suggests that the YAP pathway, which regulates the progression of cancer cells, is associated with MMP-2. In previous studies on prostate cancer development, it has been similarly demonstrated that MMP-2 is associated with invasion, metastasis, and a poor prognosis (44-46). It is theorized that ARHGAP29 may activate cell motility to upregulate MMP-2. In the present, we did not establish a direct interaction between ARGAP29 and MMP-2. Therefore, further experiments are required to support this theory.

Next, IHC staining was performed to investigate the clinical role of YAP and ARHGAP29 protein expression in prostate cancer patients. High expression levels of ARHGAP29 were related to the D’Amico risk classification, which is the risk classification of prostate cancer, and prognosis of prostate cancer patients (PSA PFS). In the present study, prostate cancer patients with high YAP or ARHGAP29 expression had a significantly poor prognosis. These differences between the TCGA database and our data may be due to different characteristics of the cohort including racial bias. Based on our data, ARHGAP29 may be a prognostic marker and therapeutic target. To confirm the present results, large-scale data analysis using Japanese samples is required in the future.

A limitation of the present study is the lack of co-localization studies of AR, YAP and ARHGAP29 in human prostate specimens as well as lack of ARHGAP29 rescue experiments in prostate cancer cells.

In conclusion, it was demonstrated that ARHGAP29 may be associated with prostate cancer cell growth and invasion as well as a clinically poor prognosis of prostate cancer patients. Therefore, ARHGAP29 may serve as a new biomarker or novel therapeutic target in prostate cancer. In a future study, the investigation of the relationship between YAP and the ARHGAP29 pathway is required to elucidate the underlying mechanism in prostate cancer.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

HMatsum, HH, MFS and HMatsuy conceived and designed the study. All authors advised the work. KS, HH and KU performed experiments. KS, MS and HH prepared the figures and drafted the original manuscript. JM, NF, YK, RI, YY, SY and TS contributed to the analysis or interpretation of the data. HMatsum, HH and HMatsuy reviewed and revised the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Institutional Ethics Committee of the Graduate School of Medicine of Yamaguchi University and written informed consent was obtained from all individuals enrolled in the study.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


