

Regulation of β -Catenin Phosphorylation by PR55 β in Adenoid Cystic Carcinoma

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Abstract. *Background/Aim:* Adenoid cystic carcinoma (AdCC) is a rare cancer of the salivary gland with high risk of recurrence and metastasis. Wnt signalling is critical for determining tumor grade in AdCC, as it regulates invasion and migration. β -catenin dephosphorylation plays an important role in the Wnt pathway, but its underlying molecular mechanism remains unclear. *Materials and Methods:* Because the regulatory subunits of protein phosphatase 2A (PP2A) drive Wnt signalling via target molecules, including β -catenin, we used qRT-PCR and immunoblot analysis to investigate the expression of these subunits in an AdCC cell line (ACCS) and a more aggressive subline (ACCS-M). *Results:* PR55 β was highly expressed in ACCS-M, suggesting its functional importance. In addition, PR55 β expression was associated with tumor grade, with ACCS-M exhibiting higher PR55 β levels. More importantly, knockdown of PR55 β in ACCS-M cells significantly reduced invasiveness and metastatic ability. Furthermore, dephosphorylation and total levels of β -catenin were dependent on PR55 β in ACCS-M. Finally, we confirmed a correlation between PR55 β staining intensity and histopathological type in human AdCC tissues. *Conclusion:* Our study provides new insight into the interaction between PR55 β and β -catenin and suggests that PR55 β may be a target for the clinical treatment of AdCC.

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Adenoid cystic carcinoma (AdCC) is a rare cancer that arises within the secretory glands, mainly the salivary gland. For head and neck AdCC, the age-adjusted incidence rate is 4.5 cases per 100,000 individuals and exhibits a slight female predominance (1). The risks for delayed recurrence and metastasis in other organs are higher in AdCC than in other oral cancers, such as squamous cell carcinoma (SCC), because of the propensity of AdCC to invade neighbouring tissues and migrate into blood vessels (2, 3). Despite the fact that hundreds of malignant AdCC tumors have been characterized at the molecular level, the underlying regulatory mechanisms of this cancer type remain unclear.

To date, many clinical AdCC studies have reported that poor prognosis is associated with pathological features such as AdCC type and grade (4). Histological specimens can be divided into three groups: tubular, cribriform, or solid. Because patients with solid AdCC are especially likely to present with distant metastases, they are the strongest candidates for further surveillance and preventative treatment (5). The use of molecular approaches to elucidate the pathways involved in invasion and migration should significantly add to our understanding of AdCC pathogenesis. Using cell sublines derived from ACCS (an AdCC cell line) by *in vivo* selection in mice, Ishii *et al.* (6) demonstrated that interactions between β -catenin and E-cadherin are associated with cell adhesion and lead to tumor metastasis. Further analysis of the molecular mechanisms involved in AdCC should contribute to the identification of potential therapeutic targets.

β -catenin is a key molecule in Wnt signalling and plays an important role in cell growth, development, and cancer. The phosphorylation of β -catenin is regulated by several kinases and phosphatases (7). In the absence of extracellular activation, phosphorylated β -catenin is degraded by proteasomes in the cytoplasm. Upon activation of the Wnt signalling pathway, these proteasomes are inhibited, and

β -catenin is able to translocate to the nucleus. Abnormal dephosphorylation of β -catenin, such as that observed in cancer development, can also result in increased nuclear translocation (8).

Protein phosphatase 2A (PP2A) is a major phosphatase that consists of a catalytic subunit (PP2Ac), structural subunit (PR65/A), and variable regulatory B subunit belonging to one of four classes (PR/B, PR/B', PR/B'', or PR/B'''). The substrate specificity of the holoenzyme is determined by the subcellular locale to which the complex is confined, the selective incorporation of the B subunit, interactions with endogenous inhibitory proteins, and specific intermolecular interactions between PP2A and target substrates (9). Mammalian cytosolic PP2A is ubiquitously expressed and is implicated in almost every signalling pathway, including those that regulate cancer metabolism, thus playing a tumor-suppressive role (9). However, PP2A has also been reported to play a cancer-promoting role, even though the underlying mechanism for this remains poorly understood (10).

Although certain B subunits of PP2A have been shown to be associated with the existence of high-grade tumors in various types of cancer, their expression levels and roles in AdCC are not yet clear. In this study, we investigated the expression levels of the different B subunits of PP2A in AdCC, as well as the role of the predominant type in the phosphorylation and translocation of β -catenin, both of which are known to be linked to tumor grade.

Materials and Methods

Ethics statement. Experiments involving human subjects were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and written consent was obtained. All protocols for the experiments in this study were reviewed and approved by the Council on Animal Care at Kyushu University (approval number A29-194-0).

Antibodies. Anti-phosphorylated β -catenin and anti- β -catenin were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- β -actin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and anti-PR55 β was obtained from Abcam (Cambridge, MA, USA).

Cell culture. Cells were cultured as described previously (11). Two AdCC cell lines were used, ACCS and ACCS-M. The latter was developed from the former by *in vivo* selection in nude mice as described previously (6) and exhibits augmented metastatic potential.

Total RNA isolation and reverse transcription. Total RNA was isolated from ACCS and ACCS-M cells. First-strand cDNA was synthesized from 3 μ g of total RNA using Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) and random hexanucleotides as primers. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification using oligonucleotide primers specific for the constitutively expressed gene β -actin (*ACTB*).

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed using the Light Cycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). The following sets of primers were used: *Ppp2r2a*, F: 5'-GCAACAGGAGATAAAGGTGGTAG-3', R: 5'-TGGTTCATGGCTCTGGAAGGTG-3'; *Ppp2r2b*, F: 5'-GCGTGATAAAGAGGCCAGAAG-3', R: 5'-TGTTGTCGCTGGCAAATACT-3'; *Ppp2r2c*, F: 5'-AGAGCTGATGACCTCACCGTTGTT-3', R: 5'-ATCAGATGAGGACACAGGCACACA-3'; *Ppp2r2d*, F: 5'-CGTGAACAAGAGAATAAAAGCCG-3', R: 5'-CTTCAATATTGGGACCCGTAG-3'; *Ppp2r3a*, F: 5'-ACGCTTGTGCAGAGGAATC-3', R: 5'-TCCAAATTCAGAGGGAGAGG-3'; *Ppp2r3c*, F: 5'-TCGTGGCGCCTAGCGACGCCAACACCTG-3', R: 5'-ATCGTCTCTCCAATCATAGGTGGTGTCTGGTGTTC-3'; *Ppp2r4*, F: 5'-GCTGAGGGGACGGCAGCCG-3', R: 5'-GCCAGATGGGTAGGACCACTGTGGCCA-3'; *Ppp2r5a*, F: 5'-GAGTATGTTTCAACTAATCGTGGTGTGTAATTGTTGAATCAGCG-3', R: 5'-TCCCATAAATTCGGTGCAGAACAGTCTTCAGG-3'; *Ppp2r5b*, F: 5'-GACAACCTGCCACTGTGCT-3', R: 5'-TCCAGCTTGTAGGAGGCTGT-3'; *Ppp2r5c*, F: 5'-GTAATAAAGCGGGCAGCAGG-3', R: 5'-CAAAGT CAAAGAGGACGCAACA-3'; *Ppp2r5d*, F: 5'-AACTCCAAGGCCACTGGA-3', R: 5'-TGCCACATCTCTCCCTTTC-3'; and *Ppp2r5e*, F: 5'-AAGCCAGACAGAAGAGGTCGCA-3', R: 5'-AGGAACAGTTCAAGCTGCTCTG-3'. Amplification was performed using the following conditions: denaturation at 95°C for 10 min, followed by 46 cycles of annealing at 60°C for 10 sec and extension at 72°C for 10 sec. Dissociation curve analyses confirmed that the signals corresponded to unique amplicons. Each experiment was performed in triplicate, and results were normalized against mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) obtained from parallel assays. Data were analysed using the LightCycler 2.0 System software package (Roche Diagnostics, Mannheim, Germany).

RNA interference. siRNA duplexes against human *Ppp2r2b* and control (scrambled) siRNA were synthesized by Eurofins Genomics (Ebersberg, Germany). The sense strands of the siRNAs were as follows: negative control, 5'-AAUUCUCCGAACGUGUCACGU-3'; *siPpp2r2b*, 5'-ACUUCCACAGCUUCACAGTT-3'.

In vitro migration assay. The number of ACCS-M cells necessary for achieving confluence in 24 h was seeded in a 6-well plate. Confluent cultures were scratched using a pipette tip to cause a wound, and images of cell migration into the wound were captured at 0, 6, 12, 18, and 24 h using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The size of the wound area was expressed using the following formula:

$$\text{Wound area (\% of control)} = \frac{\text{wound area after indicated period}}{\text{initial wound area}} \times 100 \quad (1)$$

All experiments were carried out in triplicate.

In vitro invasion assay. Cell invasion was evaluated using a 24-well Corning Matrigel Invasion Chamber (#354480; Corning Inc., Corning, NY, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were seeded in the inserts. After 48 h, invasive cells, *i.e.*, cells that had passed through the permeable support membrane, were stained with haematoxylin and then observed and counted under an optical microscope at 40 \times or 100 \times total magnification depending on cell density.

Western blotting. Cultured cells were rinsed with phosphate-buffered saline (PBS) and then lysed by sonication in sodium dodecyl sulphate (SDS) lysis buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 6% mercaptoethanol] containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The protein contents of the lysates and fractionated samples were quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein from each sample (30 μ g) were electrophoresed on 10% SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After washing with TBST [25 mM Tris-HCl (pH 8.2), 144 mM NaCl, and 0.1% Tween 20], membranes were blocked with 5% skim milk in TBST at 20–25°C. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies (DAKO, Carpinteria, CA, USA) and enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Bands were quantified using Quantity One Software (Bio-Rad Laboratories, Hercules, CA, USA) after scanning by computer-assisted densitometry (ChemIDoc XRS-J; Bio-Rad Laboratories, Hercules, CA, USA). β -actin was used as a loading control.

Immunocytochemistry. Standard protocols for immunofluorescence analysis were followed. Briefly, ACCS-M cells that had been transfected with a green fluorescent protein (GFP) expression vector were fixed with 3.7% formaldehyde and 0.2% glutaraldehyde, blocked with 5% skim milk in PBS, and incubated in anti-human β -catenin polyclonal antibody (1:100) overnight at 4°C. The next day, cells were incubated in Alexa Fluor 430-conjugated anti-rabbit IgG (1:10000; Invitrogen, Carlsbad, CA, USA) for 90 min at 37°C. The subcellular localization of Alexa Fluor 430-labelled β -catenin was determined *via* fluorescence microscopy using a Bioevo BZ-9000 microscope (Keyence, Osaka, Japan). To visualize the nuclei, cells were stained with 4',6-diamidino-2-phenylindole (DAPI).

Immunohistochemistry. For immunohistochemical analysis, 4- μ m-thick paraffin sections were dewaxed and rehydrated following standard procedures. Antigen retrieval was achieved by microwaving the sections in Target Retrieval Solution, pH 9.0 (REALTM S2367; Dako, Glostrup, Denmark) for 40 min at 160 W, followed by cooling down for 20 min at 20–25°C. Sections were washed with tap water and PBS and then treated with reagents from the Histofine Simple Stain Kit (Nichirei, Tokyo, Japan) for 30 min. After several rinses in water, sections were treated with a solution containing 0.05% 3,3'-diaminobenzidine (DAB; Merck, Darmstadt, Germany) and 0.01% H₂O₂ in 0.05 M Tris-HCl (pH 7.4) for 10 min. After several rinses in water, immunostained sections were dehydrated and cover-slipped with Malinol (Muto Pure Chemicals, Tokyo, Japan).

Statistical analysis. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A standard *t*-test was used to determine the difference in mRNA expression and in the results of the invasion and migration assays.

Results

PR55 β expression is associated with tumour grade in AdCC. To elucidate the relationship between the regulatory B subunit of PP2A and AdCC, we investigated whether AdCC tumor grade was associated with PP2A subunit

expression patterns. For this purpose, in addition to the ACCS cell line, which was obtained from human AdCC tissues, we used the ACCS-M subline, which was obtained from ACCS by transfection with a GFP expression vector and *in vivo* selection in mice. ACCS-M is characterized by increased invasiveness and metastatic potential compared with those of the parental line. These traits were confirmed by the ability of ACCS-M cells to create metastatic tumors in the submandibular glands of mice (Figure 1A). We then used qRT-PCR to compare the expression levels in ACCS and ACCS-M cells of various PP2A regulatory B subunits. We found that *Ppp2r2b*, which codes for the subunit PR55 β , was expressed at a much higher level than the genes coding for other PP2A regulatory subunits (Figure 1B). In addition, immunoblot analysis revealed that PR55 β was more highly expressed in ACCS-M than in ACCS cells (Figure 1C). Although the mRNA expression levels of *Ppp2r3a* and *Ppp2r5e* were also higher in ACCS-M cells, we could not confirm increased protein expression using immunoblot analysis (data not shown). These data suggest a relationship between invasiveness/metastatic ability and the expression of the PR55 β subunit. To verify this link, we knocked down PR55 β in ACCS-M cells using siRNA targeting its corresponding mRNA (*siPpp2r2b*). The successful suppression of PR55 β expression was confirmed by immunoblot analysis (Figure 1D). Cell migration was suppressed by the knockdown of PR55 β expression (Figure 1E). Moreover, cell invasiveness was suppressed by siRNA transfection (Figure 1F), indicating that PR55 β indeed plays an important role in AdCC malignancy.

Phosphorylation and nuclear translocation of β -catenin are regulated by PR55 β in AdCC cell lines. We hypothesized that PR55 β influences AdCC malignancy by affecting the ability of PP2A to dephosphorylate β -catenin. We investigated this hypothesis by determining β -catenin phosphorylation levels in ACCS-M cells in which PR55 β expression had been knocked down using *siPpp2r2b*. Immunoblot analysis showed that knockdown of PR55 β led to a reduction in the dephosphorylation of β -catenin (Figure 2A). Since phosphorylated β -catenin is a target of proteasome-mediated degradation, decreased dephosphorylation should result in increased degradation and lower total β -catenin levels. Indeed, immunocytochemical analysis with anti- β -catenin antibody revealed that PR55 β -knockdown cells exhibited lower β -catenin levels than control cells (Figure 2B). Interestingly, β -catenin was localized in the area around the nucleus and was also partially translocated into the nucleus. Our experiments clearly suggest that the PR55 β subunit augments the dephosphorylation of β -catenin, stabilizing the protein. Moreover, this process is critical for the cell invasion activity and metastatic potential of AdCC.

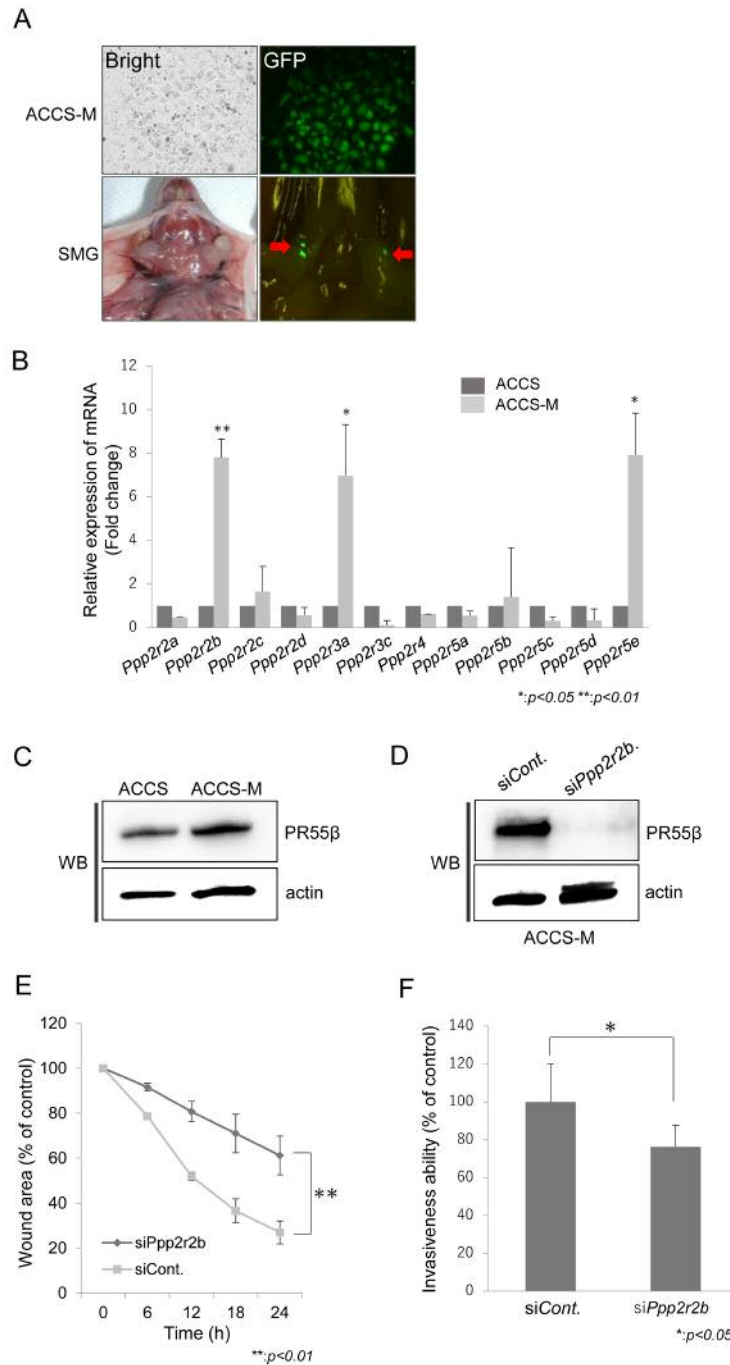


Figure 1. *PR55β* abundance is related to tumor grade in AdCC. (A) Fluorescence microscopy image of a metastatic tumor formed in the submandibular gland (SMG) of a mouse after injection of ACCS-M cells (red arrow), verifying the increased invasiveness and metastatic potential of these cells. (B) Relative mRNA levels of various regulatory B subunits in ACCS and ACCS-M cells as determined by qRT-PCR. Each bar represents the mean and standard error of five independent experiments. * $p < 0.05$, ** $p < 0.01$. (C) Western blot analysis of *PR55β* after separation of equal amounts of protein from total lysates of ACCS and ACCS-M cells using SDS-PAGE. β -actin was used as a loading control. (D) Western blot analysis of *PR55β* in total lysates of ACCS-M cells transfected with siPpp2r2b or negative control siRNA. β -actin was used as a loading control. The reduction in *PR55β* expression in the siPpp2r2b-transfected cells confirms the efficiency of the knockdown. (E) Cell migration was measured by scratching cultures with a pipette tip. Each bar represents the mean and standard error of three independent experiments. ** $p < 0.01$. (F) Cell invasion was evaluated using a 24-well Corning Matrigel Invasion Chamber. After 48 h, invasive cells, i.e., cells that had passed through the permeable support membrane, were stained with haematoxylin and then observed and counted under an optical microscope at 100 \times total magnification. Each bar represents the mean and standard error of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

PR55 β is highly expressed in AdCC. Finally, we used immunohistochemistry to investigate whether human AdCC tissues express PR55 β . Our results revealed the presence of this specific subunit in all specimens (Figure 3A). Surprisingly, specimens of squamous cell carcinoma, which is the most common type of oral cancer, were negative for PR55 β expression (data not shown). To evaluate the relationship between histopathological type and PR55 β staining intensity, immunohistochemical experiments were performed using tubular type and solid type tumors. Consistent with the prognoses of the different cell types, PR55 β staining was more intense in solid type than in tubular type tumors according to measurements using microscope software (Figure 3B). Taken together, these results strongly suggest that PR55 β is related to tumor grade in AdCC.

Discussion

Cancer cells are generally characterized by constitutive phosphorylation, and many phosphatases, including PP2A, have been found to act as tumor suppressors (12, 13). However, in this report, we have presented data indicating a tumor-promoting role of PR55 β , one of the regulatory subunits of PP2A. qRT-PCR and immunoblot analyses showed that this subunit is highly expressed in AdCC cell lines, and cell invasion and migration assays using PR55 β -knockdown cells revealed that it is necessary for both of these processes in AdCC. Moreover, immunohistochemistry confirmed its abundance in AdCC tissues, suggesting a relationship between PR55 β and tumor grade. Interestingly, a single nucleotide polymorphism (SNP) in the *Ppp2r2b* gene has been found to be associated with cancer (14). Our findings suggest that PR55 β functions as an upstream regulator of cellular invasion and migration in AdCC.

β -catenin is a critical part of the Wnt/ β -catenin signalling pathway, with roles in development, cell survival, and cancer. The dephosphorylation of β -catenin and its resulting translocation to the nucleus are associated with tumor grade in various cancers, including AdCC. Previous reports revealed interactions between β -catenin and PP2A (15-17). Moreover, it was shown that a specific regulatory subunit of PP2A, PR55 α , directly interacts with β -catenin (17). Together with our current results on the involvement of PR55 β in the Wnt/ β -catenin signalling pathway, these data suggest that the regulation of β -catenin *via* the phosphatase activity of PP2A is driven by the inclusion of regulatory subunits of the B (PR55) class, such as PR55 α and PR55 β .

In normal development, PR55 β is expressed in the foetal stage, and its expression decreases over the course of development. It is expressed mainly in the brain and testis and at low levels in the lung and spleen (18). Increased expression of PR55 β has been observed in embryos during neuronal differentiation, suggesting that it is essential for embryonic

development. In cancer, PR55 β might exert functions similar to those observed in development or differentiation. Indeed, PR55 β has been found to be related to colorectal cancer (19). However, the exact relationship between cancer and this specific subunit were previously unclear. In this report, by performing immunocytochemical analysis with PR55 β knockdown cells, we demonstrated that PR55 β promotes the dephosphorylation of β -catenin by PP2A. Moreover, our analysis showed that the dephosphorylation of β -catenin is positively correlated with total β -catenin levels. Taken together, these results suggest that PR55 β promotes tumor invasion and metastasis in AdCC by inducing the dephosphorylation of β -catenin by PP2A, subsequently reducing the proteasome-mediated degradation of β -catenin; as a result, levels of β -catenin increase, allowing it to promote invasion and metastasis. A schematic representation of this proposed mechanism is illustrated in Figure 4.

Our findings provide insights into the potential of PR55 β as a therapeutic target for this malignancy. However, further research is needed to identify the molecular mechanisms regulating the dephosphorylation of β -catenin by PP2A containing PR55 β .

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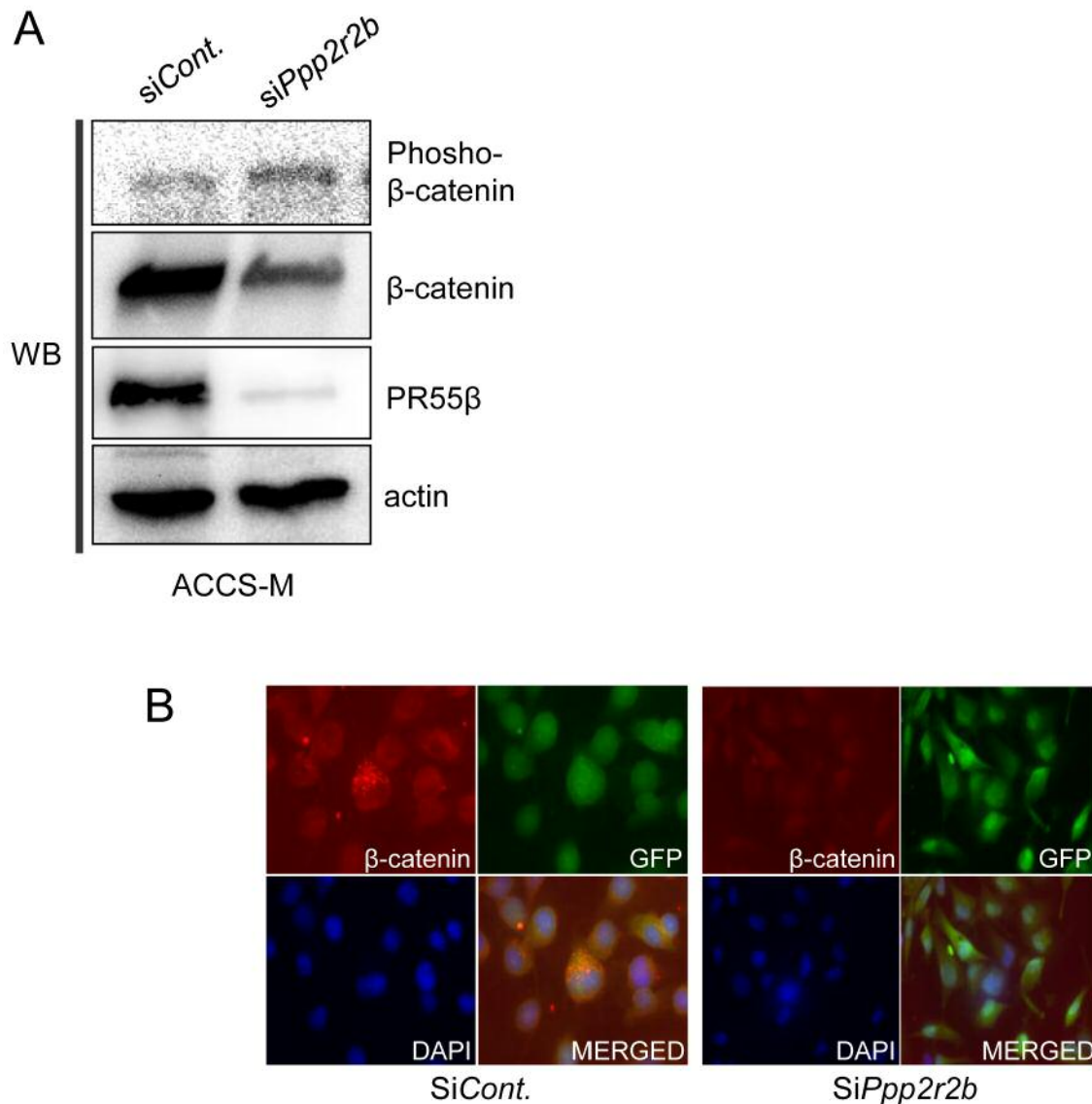


Figure 2. *PR55β* knockdown reduces both β -catenin dephosphorylation and total β -catenin levels in ACCS-M cells. (A) Dephosphorylation of β -catenin in total lysates of ACCS-M cells transfected with siPpp2r2b or negative control siRNA was examined by western blotting using an antibody recognizing phosphorylated β -catenin. (B) Immunocytochemistry analysis of total β -catenin in ACCS-M cells transfected with siPpp2r2b or negative control siRNA and fixed with 3.7% formaldehyde and 0.2% glutaraldehyde, blocked with 5% skim milk in PBS, and incubated in the indicated antibody (1:100).

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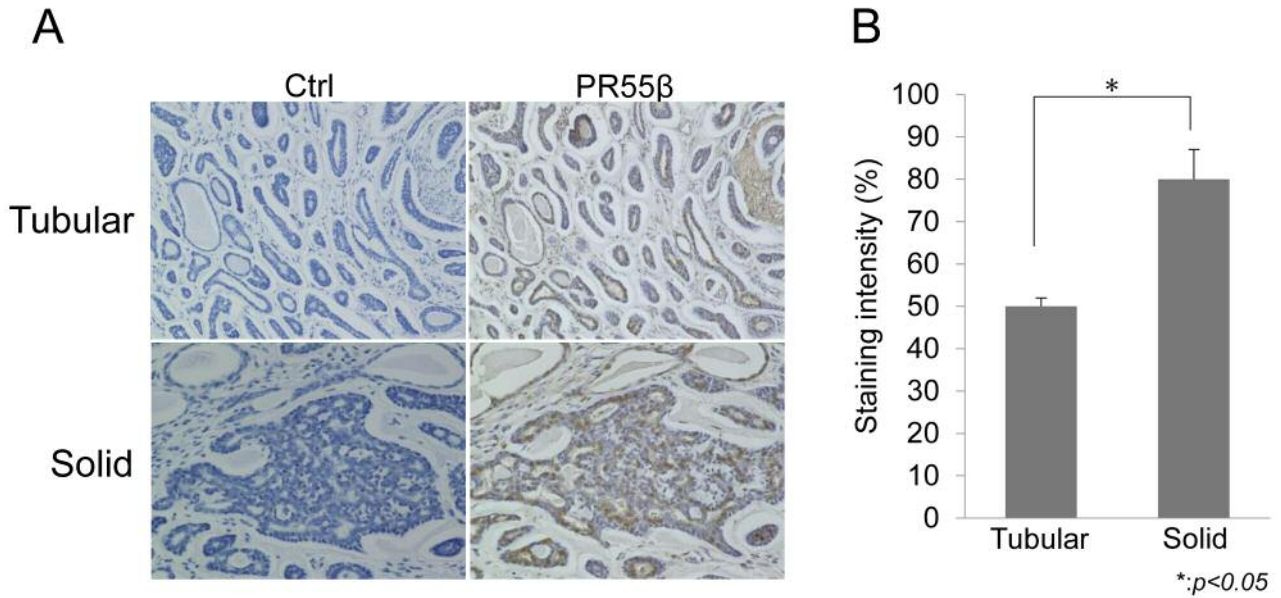


Figure 3. PR55 β expression in AdCC tissues. (A) Immunohistochemical detection of PR55 β in 4- μ m-thick paraffin-embedded sections from indicated AdCC tissues. Sections were treated with the indicated antibody overnight at 4°C, followed by DAB staining. Upper panel is tubular type and lower panel is solid type. (B) Quantitative analysis of immunohistostaining was detected via microscopy using a Biorevo BZ-9000 microscope (Keyence, Osaka, Japan). Each bar represents the mean and standard error of three independent measurements. *p<0.05.

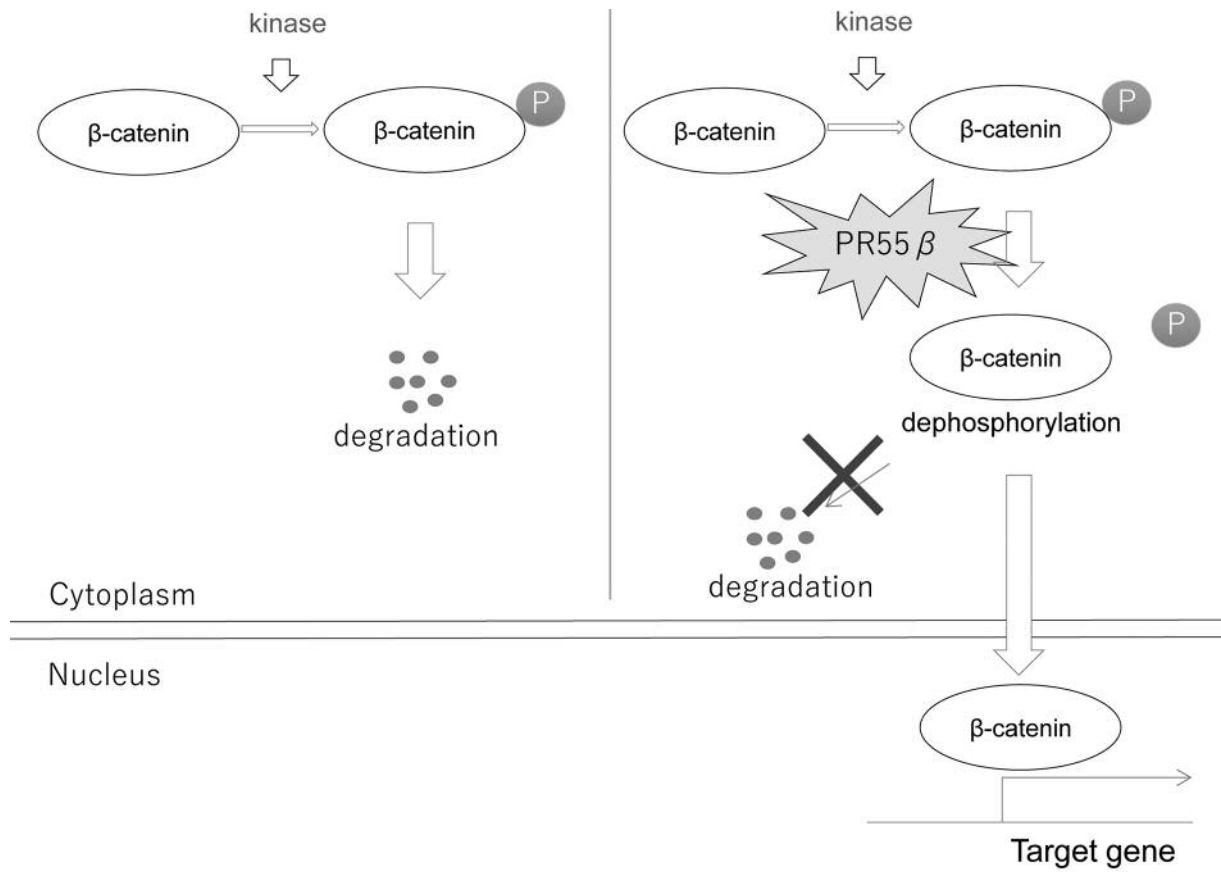


Figure 4. Hypothesis of the regulation of β -catenin dephosphorylation during cancer development.

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