(Pro)renin receptor is crucial for glioma development via the Wnt/β-catenin signaling pathway

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OBJECTIVE The (pro)renin receptor (PRR) plays an essential role in the early development of the central nervous system by activating the Wnt/β-catenin signaling pathway. The authors investigated the potential role of the PRR in the pathogenesis of glioma.

METHODS The authors performed immunohistochemical analysis to detect both the PRR and isocitrate dehydrogenase 1 with mutations involving arginine 132 (IDH1R132H) in paraffin sections of 31 gliomas. Expression of the PRR and Wnt pathway components in cultured human glioma cell lines (U251MG, U87MG, and T98G) was measured using Western blotting. The effects of PRR short interfering RNA (siRNA) on glioma cell proliferation (WST-1 assay and direct cell counting) and apoptosis (flow cytometry and the caspase-3 assay) were also examined.

RESULTS PRR expression was significantly higher in glioblastoma than in normal tissue or in lower grade glioma, regardless of IDH1R132H mutation. PRR expression was also higher in human glioblastoma cell lines than in human astrocytes. PRR expression showed a significant positive correlation with the Ki-67 labeling index, while it had a significant negative correlation with the survival time of glioma patients. Treatment with PRR siRNA significantly reduced expression of Wnt2, activated β-catenin, and cyclin D1 by human glioblastoma cell lines, and it reduced the proliferative capacity of these cell lines and induced apoptosis.

CONCLUSIONS This is the first evidence that the PRR has an important role in development of glioma by aberrant activation of the Wnt/β-catenin signaling pathway. This receptor may be both a prognostic marker and a therapeutic target for glioma.

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KEY WORDS glioma; (pro)renin receptor; Wnt/β-catenin signaling pathway; oncology

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in the adult brain and is required for appropriate neuronal differentiation and early central nervous system development via activation of the Wnt/β-catenin signaling pathway. The PRR is part of the Wnt receptor complex, and it acts as an adaptor protein between low-density lipoprotein receptor-related protein (LRP6) and vacuolar H+-adenosine triphosphatase (V-ATPase). Recent studies of PRR overexpression in breast carcinoma, pancreatic ductal adenocarcinoma, and aldosterone-producing adenoma. It has also been reported that this receptor plays a crucial role in the proliferation of pancreatic ductal adenocarcinoma cells through activation of the Wnt/β-catenin signaling pathway. Based on the aforementioned findings, the present study was conducted to test the hypothesis that PRR plays an important role in the pathogenesis of glioma through activation of Wnt/β-catenin signaling. Accordingly, we investigated PRR expression in human gliomas and its role in activating the Wnt/β-catenin signaling pathway.

### Methods

**Patients**

We obtained specimens of gliomas from 31 patients who underwent resection at Kagawa University Hospital between September 2007 and June 2015. This study was conducted in accordance with the Declaration of Helsinki and received approval from the IRB of the Kagawa University Faculty of Medicine. Written informed consent was obtained from all patients. The tumors used for immunohistochemical (IHC) studies were Grade II (diffuse astrocytoma and oligoastrocytoma), Grade III (anaplastic astrocytoma and oligoastrocytoma), and Grade IV (primary GBM, secondary GBM, and GBM with an oligodendroglioma component).

**Immunohistochemistry for PRR, IDH1 R132H, and Ki-67**

Sections were deparaffinized, soaked in 10 mM citrate buffer (pH 6.0), and heated in a microwave oven for antigen retrieval. The sections were then incubated with 5% hydrogen peroxide in methanol for 15 minutes and were blocked for 1 hour (for PRR staining) or 10 minutes (for IDH1 R132H staining) with 10% normal goat serum (Biotium) to prevent nonspecific staining. Next, the sections were incubated with an anti–human PRR rabbit polyclonal antibody overnight at room temperature, and then incubated with primary antibodies (incubation with Histofine Simple Stain MAX PO (R), Nichirei Biosciences), for 1 hour at room temperature. For negative controls, nonimmune serum was used instead of the primary antibody. Sections were washed with phosphate-buffered saline (PBS), followed by incubation with the appropriate secondary antibody [incubation with Histofine Simple Stain MAX PO (R), Nichirei Biosciences, for 10 minutes at room temperature to detect PRR, or with Histofine Simple Stain MAX PO (M), Nichirei Biosciences, for 30 minutes at room temperature to detect IDH1 R132H]. Then reaction products were detected using 3,3′-diaminobenzidine (DAB; Nichirei Biosciences). Hematoxylin was used for nuclear staining, and the sections were mounted with Malinol (Muto Pure Chemical Co.).

The percentage area of positive staining for PRR in a selected microscopic field (>40) was calculated by cellsens software (Olympus Corp.) as the IHC score (PRR IHC score).

For detection of Ki-67, sections were incubated with an anti–human Ki-67 mouse monoclonal antibody (1:50, Dako) for 20 minutes at room temperature and were stained using an I-VIEW DAB universal kit (Roche Diagnostics K.K.). To calculate the Ki-67 labeling index, Ki-67–positive cells were counted using the hotspot method, i.e., cells were counted in the most strongly stained area of the section. Immunohistochemical studies of Ki-67 were performed by the Department of Pathology at Kagawa University Hospital.

**Cell Culture**

Human GBM cell lines (U251MG, U87MG, and T98G) were obtained from the American Type Culture Collection (ATCC). These cell lines were incubated in Dulbecco’s Modified Eagle’s Medium (Life Technologies) with 10% fetal bovine serum (Japan Bio Serum), penicillin (100 U/ml), and streptomycin (100 μg/ml, Life Technologies) at 37°C under 5% CO2/95% air in a humidified incubator. Subsequently, each cell line was incubated in serum-free medium for 24 hours, after which cells were lysed with 150 μl of ice-cold lysis buffer (50 mM HEPES, 5 mM EDTA, and 100 mM NaCl, pH 7.4), 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 0.001 mM microcystin). Then the supernatant was obtained by centrifugation at 14,000g for 10 minutes at 4°C.

**Western Blotting**

Protein concentrations were measured using the method of Bradford. Total protein (30 μg) was separated using 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with LI-COR blocking solution at room temperature, and then incubated with primary antibodies at 1:1000 dilution in blocking solution (anti–PRR rabbit polyclonal antibody, Wnt2 [Santa Cruz Biotechnology], anti–active-β-catenin antibody [Millipore], and cyclin D1 antibody [Cell Signaling Technology]). After incubation with secondary antibodies (1:1000 dilution in blocking solution) coupled to infrared dyes (IRDye 800 goat anti–rabbit immunoglobulin G [IgG]; IRDye 680 goat anti–mouse IgG; and IRDye 680 donkey anti–goat IgG), protein expression was detected using an Odyssey scanner (LI-COR). To confirm equal protein loading, the membranes were reprobed with an antibody for β-actin (Sigma-Aldrich).

**Transfection With siRNA**

U251MG, U87MG, and T98G cells were incubated for 24 hours at 37°C in serum-free conditions in a humidified incubator under 5% CO2/95% air, after which transfection with PRR (ATP6AP2) and scrambled short interfering RNA (siRNA) were performed. A predesigned siRNA for...
PRR was obtained (Stealth Select RNAi, Life Technologies). According to the manufacturer’s protocol, each cell line was transfected with 240 pmol of this siRNA using Lipofectamine RNAi MAX (Life Technologies) and Opti-MEM I Reduced Serum Medium (Life Technologies). Transfection with scrambled siRNA (Stealth RNAi Negative Control, Life Technologies) was also performed in the same manner.

**Vector Construction**

After insertion of ATP6AP2 encoding PRR (Open Biosystem), the pOTB7 vector was digested with Bgl II (Takara Bio) and Sal I (Takara Bio), while the donor vector pENTR4 (Life Technologies) was digested with Kpn I (Takara Bio) and Xho I (Takara Bio). The digested vectors were treated with calf intestinal alkaline phosphatase (Takara Bio). DNA fragments were purified using a gel extraction kit (Promega) and were ligated together with a DNA ligation kit (TOYOBO). After ligation, the product was cleaned using a kit from Promega. *Escherichia coli* DH5α competent cells (Takara Bio) were transformed with the ligation product and plated onto Luria-Bertani broth containing 50 μg/ml kanamycin. The ATP6AP2 insert was detected by colony polymerase chain reaction. Plasmid DNA was purified from the cultures by using a mini prep (Promega).

**Vector Transfection**

U251MG cells were incubated for 24 hours at 37°C under serum-free conditions in a humidified incubator with 5% CO₂/95% air. Then transfection of the cells was performed using pENTR4 vectors with or without ATP6AP2. Cells were transfected with 2.5 μg of vector using Lipofectamine 3000 (Life Technologies) and Opti-MEM I Reduced Serum Medium (Life Technologies) according to the manufacturer’s protocol. After 72 hours, the WST-1 assay was performed and protein was harvested for Western blotting.

**WST-1 Assay and Cell Counting to Assess Cell Proliferation**

These assays were performed using the 3 human GBM cell lines. First, 5 × 10⁴ cells were seeded into the wells of 6-well plates. After incubation for more than 24 hours at 37°C with 10% fetal bovine serum in a humidified incubator under 5% CO₂/95% air, the initial number of cells was counted at time 0. Then transfection was performed with PRR siRNA and scrambled siRNA. After incubation for 48 hours, the WST-1 assay was performed according to the manufacturer’s protocol (Takara Bio) to determine proliferative activity. Premixed WST-1 reagents (100 μl) were added to each well, incubation was done for 1.5 hours at 37°C in a humidified incubator under 5% CO₂/95% air, and the absorbance of each well was measured at 450 nm with a microplate reader. The proliferative activity of PRR-expressing U251MG cells was also evaluated by the WST-1 assay, which was done as described above.

Direct cell counting was performed at 24, 48, and 72 hours after transfection. Cells were treated with 0.05% Trypsin-EDTA (Life Technologies), centrifuged, and re-suspended in PBS. To distinguish between viable and dead cells, the suspensions were stained with AccuStain (Digital Bio). Then aliquots of the cell suspensions (10 μl) were loaded into an AccuChip 4x Counting Kit (Digital Bio) and viable cells (per ml) were counted using an automated cell counter (ADAM-MA, Digital Bio).

**Detection of Apoptosis Using Annexin V and Propidium Iodide**

After transfection with PRR or scrambled siRNA, U251MG cells were incubated for 120 hours. Then the cells were treated with 0.05% Trypsin-EDTA (Life Technologies), centrifuged, washed twice in PBS, and resuspended in 1X annexin-binding buffer according to the manufacturer’s protocol (Life Technologies). Next, the cells were stained with fluorescein isothiocyanate (FITC)–labeled annexin V and 100 μg/ml propidium iodide (PI) working solution for 45 minutes at room temperature, after which 400 μl of 1X annexin-binding buffer was added. Flow cytometry was performed with monitoring of emission at 525 nm (FITC) and 675 nm (PI).

**Detection of Apoptosis by the Caspase-3 Assay**

At 48 hours after transfection with PRR or scrambled siRNA, caspase-3 activity was detected according to the manufacturer’s protocol (Medical & Biological Laboratories). Reaction buffer (2×) and caspase-3 substrate were added to cell lysates, which were injected into the wells of 96-well plates and incubated at 37°C for 2 hours (U87MG cells) or 12 hours (U251MG and T98G cells). Then the absorbance was measured at 405 nm with a microplate reader. Caspase-3 activity in each well was calculated from a standard curve and normalized by the total protein concentration.

**Statistical Analysis**

All analyses were performed using GraphPad Prism (version 6.0 for Windows, GraphPad Software). One-way ANOVA was performed using a post hoc Scheffe’s test to analyze the PRR IHC score, the Ki-67 labeling index, and proliferative activity (WST-1 assay) of tumors in each WHO grade. Two-way ANOVA was applied to determine the effect of transfection with PRR siRNA and the incubation period on cell numbers in human GBM cell lines. The correlation between PRR IHC score and the Ki-67 labeling index was analyzed using Spearman’s rank-correlation coefficient test. The Kaplan-Meier method was used to plot the survival curves of 2 groups of glioma patients categorized by the PRR IHC score.

**Results**

**PRR Expression in Human Gliomas of Each WHO Grade**

Immunohistochemistry revealed strong cytoplasmic PRR expression by GBM cells, while PRR expression was considerably weaker in normal tissues (Fig. 1A). In addition, PRR expression was higher in lysates from the 3 human GBM cell lines than in the lysate of primary astrocytes (Fig. 1B). These results demonstrated a high level of PRR expression by GBM lesions.
PRR expression was also confirmed in gliomas of each WHO grade (Fig. 1C). Table I summarizes the demographic and clinicopathological characteristics of the 31 glioma patients. The level of IHC expression of PRR was expressed as a score (PRR IHC score) calculated by a software program (cellSens, Olympus Corp.), with the mean score being 0.24% (range 0.11%–0.46%) for normal tissue, 2.8% (range 1.59%–3.78%) for Grade II glioma, 8.4% (range 6.25%–9.54%) for Grade III glioma, and 21.2% (range 13.33%–30.99%) for Grade IV glioma (Table I). PRR expression was detected in both primary and secondary GBM, but only primary GBM was negative for IDH1R132H (Fig. 1C). PRR expression was significantly higher in GBM than in lower grade gliomas and normal tissues (Fig. 1D). There was no significant difference in PRR IHC score between the tumors with and those without IDH1R132H (Supplementary Fig. S1; Mann-Whitney U-test, p = 0.3192). Collectively, these findings demonstrated that PRR expression increased along with the WHO grade regardless of the presence/absence of IDH1R132H.

**Relationship Between PRR Expression and the Ki-67 Labeling Index or Survival**

Grade IV tumors showed stronger Ki-67 immunoreactivity than Grade II and Grade III tumors (Fig. 2A). There was a significant positive correlation between the PRR
IHC score and the Ki-67 labeling index (r = 0.8080, p < 0.0001), and both of these parameters differed according to the WHO grade (Fig. 2B). In addition, GBM had a significantly higher Ki-67 labeling index than lower grade gliomas (Fig. 2C). Taken together, these data indicated that PRR expression was significantly correlated with cell proliferation as shown by the Ki-67 labeling index.

There was a significant negative correlation between the PRR IHC score and the survival time of the glioma patients (r = -0.6499, p = 0.0002) (Fig. 2D). While there was no significant difference in PRR expression between Grade II and Grade III tumors, PRR expression was significantly higher in GBM than in lower grade tumors (Fig. 1D). Based on these data, we set a cutoff value of 10% for the PRR IHC score and used the Kaplan-Meier method to compare the cumulative survival of 2 groups based on this cutoff value. This analysis demonstrated that patients with high PRR IHC scores had shorter survival than patients with low scores (Fig. 2E).

Effect of PRR Expression on Wnt/β-Catenin Signaling in Human GBM Cell Lines

It has been reported that loss of endogenous Wnt2 and β-catenin suppresses cell growth and induces apoptosis in glioma cell lines. Therefore, we examined whether PRR had an influence on the Wnt/β-catenin signaling pathway in human GBM cell lines. Expression of Wnt2, active β-catenin, and cyclin D1 (representative components of the pathway) was confirmed after treatment with the vehicle and scrambled siRNA. Treatment with PRR siRNA significantly reduced PRR expression by all 3 GBM cell lines compared with scrambled siRNA. PRR siRNA also significantly decreased expression of Wnt2, active β-catenin, and cyclin D1 in all 3 cell lines (Fig. 3). On the other hand, PRR overexpression increased Wnt2 expression in U251MG cells compared with transfection of the empty plasmid vector (Mock) (Supplementary Fig. S2 [A]).

Loss of PRR Induces Apoptosis of GBM Cell Lines

Flow cytometry and caspase-3 assaying were performed to evaluate apoptosis. FITC-labeled annexin V identifies early apoptotic cells by binding to phosphatidylserine, which undergoes translocation from the inner to outer leaflet of the plasma membrane, while PI detects cells in the late stage of apoptosis. Thus, viable cells in the early stage of apoptosis can be distinguished from viable cells in the late stage of apoptosis.
late-stage cells and dead cells by double labeling with annexin V and PI. At 120 hours after transfection with PRR siRNA, there was a significantly lower percentage of viable cells compared with after transfection with scrambled siRNA (73.2% vs 86.3%, respectively) (Fig. 5A). Hence, there was a significant increase in the proportion of early apoptotic cells, late apoptotic cells, and dead cells after transfection with PRR siRNA (Fig. 5B).

Similar data were obtained by flow cytometry. That is, treatment with PRR siRNA significantly increased caspase-3 activity in all 3 cell lines (Fig. 5C–E). Taken together, these results demonstrated that loss of PRR induced apoptosis of human GBM cell lines.

Discussion

The present study revealed several new points regarding the role of the PRR in Wnt/β-catenin signaling–dependent development of glioma. First, an aberrant increase of PRR expression was confirmed in WHO Grade II–IV gliomas irrespective of the presence or absence of IDH1R132H mutation, and the level of PRR expression was directly proportional to the WHO grade. Second, PRR expression had a positive correlation with the Ki-67 labeling index, and glioma patients with high PRR expression showed shorter survival. Third, we demonstrated that PRR induced proliferation of human glioma cells through activation of the Wnt/β-catenin signaling pathway. Fourth, we showed that PRR suppression with siRNA induced apoptosis of human glioma cells via activation of caspase-3.

The first finding was an aberrant increase of PRR expression in WHO Grade II–IV gliomas, with PRR expression being proportional to the grade. IDH1 mutations are
FIG. 3. Effect of PRR siRNA on Wnt/β-catenin signaling in human GBM cell lines. Effect of PRR siRNA on expression of Wnt2, active β-catenin, and cyclin D1 in U251MG cells (left) and U87MG cells (right). Silencing of PRR led to reduced expression of these components of the Wnt/β-catenin pathway. Similar results were obtained in 3 experiments. β-actin was used as the loading control.

FIG. 4. Effect of PRR siRNA on GBM cell proliferation. A–C: PRR expression and the WST-1 proliferation assay in 3 human GBM cell lines (U251MG cells [A], U87MG cells [B], and T98G cells [C]) at 48 hours after transfection (mean ± SEM, n = 3). β-actin was used as the loading control. ***p < 0.001; **p < 0.01. D–F: Effect of PRR siRNA transfection on total cell numbers (U251MG cells [D], U87MG cells [E], and T98G cells [F]) determined by direct cell counting (mean ± SEM, n = 3). Treatment with PRR siRNA prevents an increase in cell numbers. *p < 0.05 vs scrambled siRNA; **p < 0.05 vs vehicle at time zero; ***p < 0.05 vs PRR siRNA at time zero.
detected in more than 80% of Grade II and III gliomas, and 55%–85% of secondary GBM tumors that have undergone transformation from Grade II or III glioma express IDH1 mutations. On the other hand, less than 10% of primary GBM tumors not arising from Grade II or III glioma have IDH1 mutation. We detected PRR overexpression in all of the gliomas that we examined irrespective of the presence or absence of IDH1 R132H, which suggests that PRR expression increases before genetic alteration of IDH1 R132H. Thus, PRR may promote the development of glioma through a different molecular mechanism from that of IDH1 R132H. However, the earliest stage of PRR overexpression remains unclear because there were no WHO Grade I pilocytic astrocytomas in the present series, and thus further investigation will be needed to identify the initial stage of PRR overexpression during glioma development.

Previous studies have indicated that the Ki-67 labeling index increases with the progression of glioma and with worse overall survival, suggesting that this index is an important prognostic factor for glioma patients. In the present study, PRR expression was found to have a significant positive correlation with the Ki-67 labeling index, suggesting that increased expression of PRR contributes to proliferation of glioma cells. In addition, Kaplan-Meier analysis showed that glioma patients with a high PRR IHC score (≥ 10%) had a shorter survival time than patients with a low IHC score (< 10%), suggesting that PRR may be useful for predicting survival. Thus, PRR expression may not only be an indicator of tumor progression, but may also be a prognostic factor for glioma independent of the IDH1 mutation status.

The Wnt/β-catenin signaling pathway plays an important role in the development of glioma. Wnt2 is one of the Wnt ligands and it acts as a regulator of the canonical Wnt/β-catenin signaling pathway. In the present study, expression of Wnt2 was not detected in normal brain tissue. However, Wnt2 expression was prominent in gliomas, and its level of expression was positively correlated with the WHO tumor grade. It has been reported that knockdown of Wnt2 by treatment with siRNA inhibits proliferation of the human U251MG GBM cell line and induces apoptotic cell death. It seems that the expression of Wnt2 and PRR is synchronized in glioma. We also observed that treatment with PRR siRNA significantly decreased the expression of Wnt2, active β-catenin, and cyclin D1 by human GBM cell lines. Conversely, Wnt2 expression was increased in glioma cells with PRR overexpression. Although this clearly indicates that the Wnt pathway lies downstream of the PRR, we were not able to obtain direct evidence of the relationship between this receptor and the Wnt pathway, since the inhibition of cell proliferation induced by PRR siRNA was not fully reversed through transfection with constitutively activated β-catenin. Accordingly, further investigation is needed using a molecular pathway other than the Wnt/β-catenin signaling pathway. Although the precise molecular mechanism mediating the interaction between PRR and Wnt2 is still unclear, these data support the hypothesis that...
the PRR plays a crucial role in regulating cell proliferation and apoptosis in glioma through the Wnt/β-catenin signaling pathway, as was recently demonstrated in pancreatic cancer.32

This study also demonstrated that PRR expression in tumor tissues increased in proportion to the WHO grade. Higher grade gliomas are more likely to contain a population of glioma stem cells (GSCs) than lower grade gliomas.22,30 GSCs exhibit many of the properties of stem cells, including self-renewal potential, extended proliferation, and differentiation into multiple cell types mediated via the Wnt/β-catenin signaling pathway.1,5,8,12,15,23,33,34 Recent studies have shown that V-ATPase contributes to reduction in sensitivity to chemotherapy.4,17,29 In addition, it was reported that the GI subunit of V-ATPase is required for proliferation of GSCs, suggesting that pH imbalances contribute to maintenance of the GSC niche.7 Since the PRR regulates both V-ATPase and the Wnt/β-catenin signaling pathway, it may be involved in modulating GSC function. Accordingly, the role of the PRR in regulation of GSCs should be investigated in the future.

Conclusions

The present study revealed that the PRR plays an important role in the development of glioma by aberrant activation of the Wnt/β-catenin signaling pathway. The PRR may be a potential prognostic indicator as well as a therapeutic target for gliomas of all grades.

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Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: Kouchi, Shibayama, Ogawa, Miyake, Nishiyama. Acquisition of data: Kouchi, Shibayama. Analysis and interpretation of data: Kouchi, Shibayama, Ogawa, Miyake, Nishiyama. Drafting the article: Kouchi, Shibayama, Nishiyama. Reviewed submitted version of manuscript: Kouchi, Shibayama, Nishiyama, Tamiya. Approved the final version of the manuscript on behalf of all authors: Kouchi. Statistical analysis: Kouchi, Shibayama. Study supervision: Tamiya.

Supplemental Information
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