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ORIGINAL PAPER

Involvement of aquaporin-5 in differentiation of human gastric cancer cells

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Abstract Litttle is known about the function of aquaporin (AQP) water channels in human gastric cancer. In the upper or middle part of human stomach, we found that expression level of AQP5 protein in intestinal type of adenocarcinoma was significantly higher than that in accompanying normal mucosa. AQP5 was localized in the apical membrane of the cancer cells. On the other hand, both AQP3 and AQP4 were not up-regulated in the adenocarcinoma. To elucidate the role of AQP5 in cancer cells, AQP5 was exogenously expressed in a cell line of poorly differentiated human gastric adenocarcinoma (MKN45). The AQP5 expression significantly increased the proportion of differentiated cells with a spindle shape, the activity of alkaline phosphatase, a marker for the intestinal epithelial cell type of cancer cells, and the expression level of laminin, an epithelial cell marker. Treatment of the MKN45 cells stably expressing AQP5 with $HgCl_2$, an inhibitor of aquaporins, significantly decreased the proportion of differentiated cells and the activity of alkaline phosphatase. Our results suggest that up-regulation of AQP5 may be involved in differentiation of human gastric cancer cells.

Keywords Aquaporins · Stomach · Cancer cells

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Introduction

Aquaporins (AQPs) are integral membrane proteins which facilitate the movement of water, and they are expressed in many kinds of cells especially in polarized epithelial cells. Recently, it has been reported that AQPs contribute to differentiation of non-cancer cells. In osteoclast-lineage cells such as mice bone marrow macrophages and the murine macrophage-like cell line (RAW264.7), AQP9 was expressed in the cells as the only aquaporin and was found to be involved in osteoclast differentiation, specifically in fusion process [1]. In Madin-Darby canine kidney (MDCK) epithelial cells, it has been reported that basolateral AQP3 accumulates with E-cadherin precisely at the site of initial cell–cell adhesion [2].

Three AQP isoforms (AQP3, 4 and 5) have been reported to be expressed in glandular cells in the rat stomach. AQP3 is localized in the basolateral membrane of

surface mucous cells [3], AQP4 in the basolateral membrane of parietal cells [4, 5] and AQP5 in the apical membrane of pyloric gland secretory cells [6, 7].

So far, the expression of AQPs in human gastric cancer tissues and the role of AQP5 expression in gastric cancer cells have not been reported. In the present study, we report a significant increase of AQP5 expression in the upper or middle part of stomach tissues obtained from patients with intestinal type of gastric adenocarcinoma. We also report the effects of exogenous expression of AQP5 in a cell line of poorly differentiated human gastric adenocarcinoma on the cell differentiation.

Materials and methods

Tissue procurement

Human gastric carcinoma specimens in the upper or middle part of stomach were obtained from surgical resection of Japanese patients at University of Toyama Hospital in accordance with the recommendations of the Declaration of Helsinki and with the ethics committee approval. All patients gave informed consent. In all cases, the control specimens were collected from accompanying normal mucosa, which were 5 cm apart from the carcinoma. The cancer tissue and the normal epithelial layer were isolated from the resected stomach with scissors and forceps. These samples were free from the serosa and muscularis propria. The blood vessels around the tissues were carefully removed.

The clinical and histological classifications were carried out by expert pathologists according to the TNM classification [8] and the Japanese Classification of Gastric Carcinoma edited by Japanese Gastric Cancer Association (The 13th edition).

Immunohistochemistry

Immunohistochemical studies of human gastric tissues were performed on formalin-fixed and paraffin-embedded tissue section. The sections (5 μm-thick) were deparaffinized in xylene and rehydrated through graded series of ethanol. Antigen retrieval was performed using a pressurized heating chamber (Pascal, DakoCytomation, Carpinteria, CA, USA) and Target Retrieval Solution (pH 9.0, DakoCytomation) at 120°C for 4 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 solution containing 0.1% sodium azide for 5 min at room temperature, and non-specific blocking was performed with blocking reagent (DakoCytomation) for 10 min at room temperature. The sections were incubated with the anti-AQP5 (C-19) antibody (1:100 dilution) (Santa Cruz, CA, USA) for 12 h at 4°C, and then with the secondary antibody (Histofine MAX-PO(G); Nichirei Bioscience Inc., Tokyo, Japan) for 45 min at room temperature. The immunoreactive product was visualized with 3,3′-diaminobenzidine (DakoCytomation). The sections were then counterstained with hematoxylin. For negative control sections, one volume of the primary antibody was pre-incubated with four volumes of the corresponding blocking peptide.

Exogenous expression of AQP5 in a poorly differentiated human gastric adenocarcinoma cell line. A full-length cDNA encoding human AQP5 was inserted into the pcDNA4/His vector (Invitrogen, Carlsbad, CA, USA). MKN45, a poorly differentiated human gastric adenocarcinoma cell line, was maintained in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Eqitech-Bio, Kerrville, TX, USA). For transient expression of AQP5, the cells were transfected with the AQP5-pcDNA4/His vector (AQP5-transfected cells) or pcDNA4/His vector (mock cells) using Lipofectamine 2000 (Invitrogen). The transfection was performed at 24 h after the cell seeding and the transfected cells were cultured for 48 h. For stable expression of AQP5, the MKN45 cells were transfected with the AQP5-pcDNA5/TO vector using Lipofectamine 2000 and cultured for 24 h. The transfected cells were selected in the presence of 1,000 units/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan).

Western blotting

Membrane fractions of the human gastric tissues and a human gastric cancer cell line (MKN45) were prepared [9], and Western blotting was performed [10]. The blotting was performed with 30 μg of membrane protein. Signals were visualized with the ECL Plus system (GE Healthcare, Buckinghamshire, UK). Anti-AQP3 (C-18) and anti-AQP5 (C-19) antibodies (Santa Cruz) were used at 1:200 dilution and anti-AQP4 antibody (H-19; Santa Cruz) was used at 1:1,000 dilution. For negative control, one volume of each primary antibody was pre-incubated with five volumes of the corresponding blocking peptide. Anti-β-actin (Sigma-Aldrich, St. Louis, USA) and anti-laminin β3 (H-300; Santa Cruz) antibodies were used at 1:500 dilution. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat IgG was used as a secondary antibody (1:2,000 dilution).

Immunocytochemistry

MKN45 cells were fixed with ice-cold methanol for 7 min and permeabilized with phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 0.1% BSA for 15 min at room temperature. Non-specific blocking was performed with 3% BSA. The permeabilized cells were incubated

Table 1 Clinical characteristics of the tissues of gastric adenocarcinomas

Case no.	Age	Sex	Location	Histological type (grading)	TNM classification	*H. pylori* infection
1	74	M	U	Tubular (mod)	II	(+)
2	81	M	U	Tubular (well)	IB	ND
3	69	M	M	Tubular (well)	IA	(+)
4	68	M	U	Tubular (well)	II	ND
5	67	F	U	Tubular (mod)	IIIA	ND
6	73	M	U	Tubular (mod)	II	ND
7	51	M	M	Tubular (mod)	IB	(+)
8	76	F	U	Tubular (mod)	IV	ND
9[a]	61	M	U	Tubular (mod)	II	ND
10	55	M	U	Tubular (poor)	IIIA	ND
11	50	M	U	Tubular (poor)	IV	ND
12	67	F	U	Tubular (poor)	IB	(−)
13	55	M	U	Mucinous	IB	ND
14	86	M	M	Tubular (poor)	II	(−)
15	47	M	U	Signet ring cell, tubular (poor)	IA	ND
16	67	M	M	Signet ring cell, tubular (poor)	IA	(+)
17	66	F	M	Tubular (poor)	IA	(+)
18	51	M	U	Signet ring cell carcinoma	IA	(+)
19	70	M	M	Tubular (poor)	IIIA	(+)

U upper area of the stomach, *M* middle area of the stomach, *well* well differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma, *poor* poorly differentiated adenocarcinoma, *Case No. 1 to 9* intestinal type of Laurén classification, *case nos. 10 to 19* diffuse type of Laurén classification, *case nos. 15 and 16* combined lesion, *ND* not determined, + positive, − negative

[a] Multifocal cancer

with the anti-AQP5 and anti-laminin β3 antibodies (1:100 dilution) for 12 h at 4°C, and then with the Alexa Fluor 488-conjugated and Alexa Fluor 546-conjugated anti-IgG antibodies (1:100 dilution; Invitrogen) for 1 h at room temperature. Immunofluorescence images were visualized by using a Keyence BZ-8000 microscope.

Cell differentiation assay

The photo images under a microscope of 6–7 independent areas were randomly obtained in one experiment. Cell differentiation was assessed by counting the number of differentiated cells that have a spindle-like shape and larger diameter in an area (100 cells). Three experiments on different days were performed. Then, the mean number of differentiated cells from 10 to 20 independent areas (the number of differentiated cells per 100 cells) was calculated.

Cell proliferation assay

Cell proliferation was assessed by counting the total number of cells in a 12-well culture plate. In each well, 1×10^5 cells were seeded and cultured for 3 days in the RPMI-1640 medium supplemented with 10% FBS. The mean cell number from three independent wells was obtained in one experiment, and the mean values from 3 to 6 experiments were averaged.

Measurement of alkaline phosphatase activity

MKN45 cells were lysed with the solution containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA and 50 mM Tris–HCl (pH 7.6). The lysate was centrifuged at $15{,}000 \times g$ for 15 min at 4°C, and the supernatant was used for the following assay. Alkaline phosphatase activity of the cell lysate was measured in 750 mM 2-amino-2-methyl-1-propanol buffer solution (pH 10.3, Sigma-Aldrich, St. Louis, USA) containing 50 μg protein and 7.5 mM *p*-nitrophenyl phosphate. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 1 N NaOH solution. *p*-Nitrophenol concentration in the solution was determined from the absorbance at 405 nm.

Statistics

Results are shown as means ± SE. Differences between groups were analyzed by one-way ANOVA. Comparison

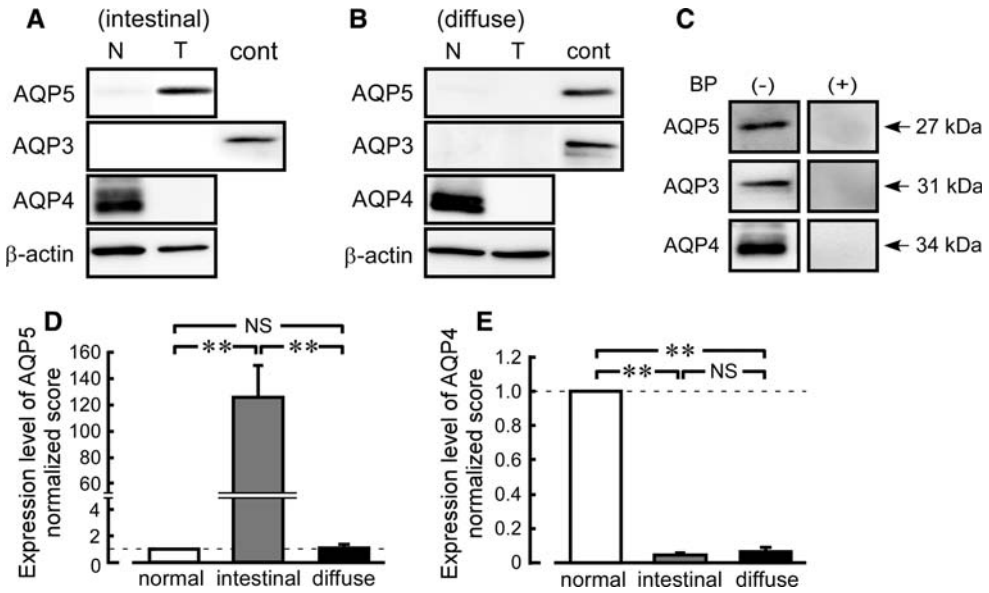

Fig. 1 Expression of AQP3, 4 and 5 proteins in intestinal and diffuse types of human gastric adenocarcinomas. **a**, **b** Western blotting for detecting proteins of AQP5 (27 kDa), AQP3 (31 kDa) and AQP4 (34 kDa) in paired normal mucosa (N) and adenocarcinoma (T). Typical example of intestinal type of adenocarcinoma (**a**; patient No. 4 in Table 1) and diffuse type of adenocarcinoma (**b**; patient No. 10 in Table 1) are shown. As a loading control, expression level of β-actin (42 kDa) was tested. Membrane fraction of rat kidney was used as a positive control for AQP3 (cont in **a** and **b**) and membrane fraction of intestinal type of human gastric adenocarcinoma (No. 6 in Table 1) as a positive control for AQP5 (cont in **b**). **c** The specificity of bands of 27 kDa (AQP5), 31 kDa (AQP3) and 34 kDa (AQP4) was confirmed by using the corresponding blocking peptides (BP). **d**, **e** The level of protein expression of AQP5 (**d**) and AQP4 (**e**) in intestinal and diffuse types of adenocarcinomas was compared with that of paired normal mucosa. The score for the normalized expression level of AQP5 or AQP4 = [(amount of AQP5 or AQP4 protein in the adenocarcinoma)/(amount of β-actin protein in the adenocarcinoma)]/[(amount of AQP5 or AQP4 protein in the normal mucosa)/(amount of β-actin protein in the normal mucosa)]. The score for normal mucosa was normalized as 1. Averaged score ± SE of the intestinal and diffuse types of adenocarcinomas are shown. $n = 10$. $^{**}p < 0.01$. *NS* not significant ($p > 0.05$)

between the two groups was made by using Student's *t*-test. Statistically significant differences were assumed at $p < 0.05$.

Results

Up-regulation of AQP5 in intestinal type of human gastric adenocarcinoma

Expression of AQPs in human gastric tissues of adenocarcinomas and accompanying normal mucosa from upper or middle part of stomach of the patients were studied. According to Laurén classification, all of the well and moderately differentiated adenocarcinomas (No. 1–9) were judged as "intestinal type" and all of the poorly differentiated adenocarcinomas, mucinous adenocarcinomas and signet ring cell carcinomas (No. 10–19) were judged as "diffuse type" (Table 1).

Figure 1a shows typical Western blots for detecting the proteins of AQP3, AQP4 and AQP5 in the membrane fractions prepared from intestinal type of adenocarcinoma. Interestingly, extensive increase in the expression level of AQP5 protein (27 kDa) was observed in 10 out of 10 carcinomas (9 patients) compared with the AQP5 level in the accompanying normal tissues, and this increase was significant (Fig. 1a, d). In contrast, the decrease in the expression of AQP4 protein (34 kDa) was observed in 10 out of 10 carcinomas (Fig. 1a, e). The high level expression of AQP4 in the normal mucosa seems to be attributed to the gastric parietal cells as previously described [4, 11]. No significant expression of AQP3 protein was observed in all gastric tissues tested (Fig. 1a), although a low level of AQP3 expression was reported in gastric pits by using human tissue microarrays [12]. Using the corresponding blocking peptides, we confirmed the specificity of anti-AQP3 antibody for the 31-kDa band, anti-AQP4 antibody for the 34-kDa band and anti-AQP5 antibody for the 27-kDa band (Fig. 1c).

Next, we examined expressions of proteins of AQP3, AQP4 and AQP5 in diffuse type of adenocarcinoma. No significant increase in the expression level of AQP5 was observed in 10 out of 10 carcinomas (Fig. 1b, d), which is in contrast to the case of intestinal type of adenocarcinoma. The decrease in the expression level of AQP4 was observed in 10 out of 10 carcinomas (Fig. 1b, e). The

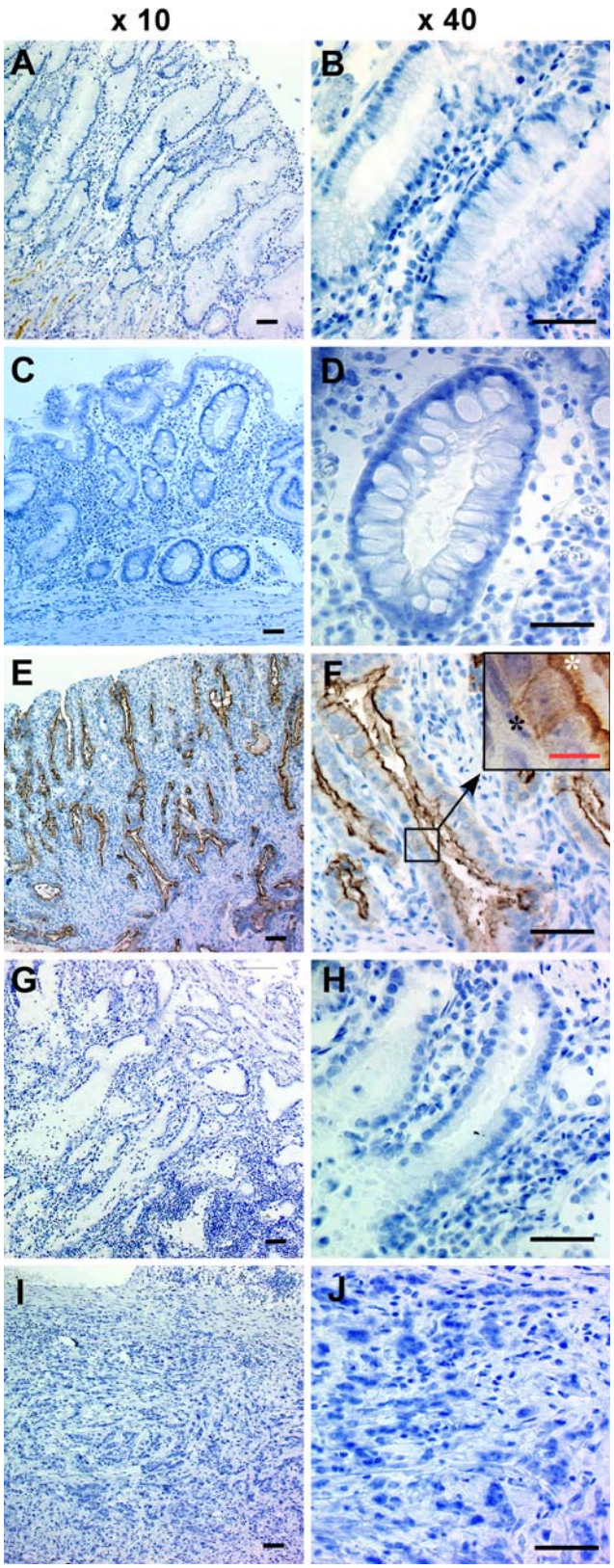

Fig. 2 Immunostaining for AQP5 in tissues of the carcinoma and accompanying normal mucosa. Tissue sections were immunostained with the anti-AQP5 antibody. The portions stained with *brown colors* indicate the expression of AQP5. Representative pictures obtained from three independent experiments are shown. Original magnification ×10 (*left panels*) and ×40 (*right panels*). Tissues were obtained from patients No. 2 (**a**–**h**) and No. 11 (**i**, **j**) in Table 1. No staining in normal tissues (**a**, **b**) and intestinal metaplasia (**c**, **d**). Strong staining in intestinal type of adenocarcinoma (**e**, **f**) but no staining in the presence of the blocking peptide (**g**, **h**). In (**f**), a *white asterisk* shows the apical side of cells and a *black asterisk* shows the basal side of cells. No staining in diffuse type of adenocarcinoma (**i**, **j**). *Scale bars* 50 μm (*black color* **a**–**j**) and 10 μm (*red color* **f**)

expression of AQP3 was observed neither in the cancer tissue nor normal mucosa (Fig. 1b).

Localization of AQP5 in intestinal type of gastric adenocarcinoma

In the paraffin-embedded tissue sections, the cells in intestinal type of cancer tissues showed clear immunoreactivity for AQP5 (Fig. 2e, f). AQP5 was localized in the apical membrane of the cancer cells (Fig. 2f). The specificity of anti-AQP5 antibody for this positive staining was confirmed by using the blocking peptide (Fig. 2g, h). The immunoreactivity was weak in normal accompanying gastric cells (Fig. 2a, b) and in diffuse type of cancer tissues (Fig. 2i, j), which are in accordance with the results shown in Fig. 1. It is noted that no significant expression of AQP5 was observed in the tissue of intestinal metaplasia (Fig. 2c, d).

Cell differentiation induced by the AQP5 expression in a gastric cancer cell line

To speculate the function of AQP5 in gastric cancer cells, AQP5 was exogenously expressed in MKN45, a poorly differentiated human gastric adenocarcinoma cell line. A significant expression of AQP5 protein was observed in 71 ± 1% of the cells transfected with the AQP5-pcDNA4/His vector ($n = 5$) (Fig. 3a, b). But no significant expression of AQP3 or AQP4 was observed in mock and the AQP5-transfected cells (Fig. 3c).

In Fig. 4, we checked whether the expression of AQP5 in MKN45 cells induced cell differentiation. First, as a positive control, AQP5-free MKN45 cells were treated with sodium butyrate (SB), which inhibits cell proliferation and stimulates cell differentiation. It has been reported that SB inhibits histone deacetylase, which allows histone hyperacetylation. Such hyperacetylation leads to transcription of several genes, including the cyclin-dependent kinase inhibitory protein p21/Cip1. The induction of p21/Cip1 accounts for cell arrest in the G1 phase of the cell cycle [13]. Here, no significant expression of AQP5 was observed in the SB-non-treated or the SB-treated MKN45 cells (data not shown). Treatment with SB clearly increased the proportion of differentiated cells that have a spindle-like shape and larger diameter (Fig. 4a, b).

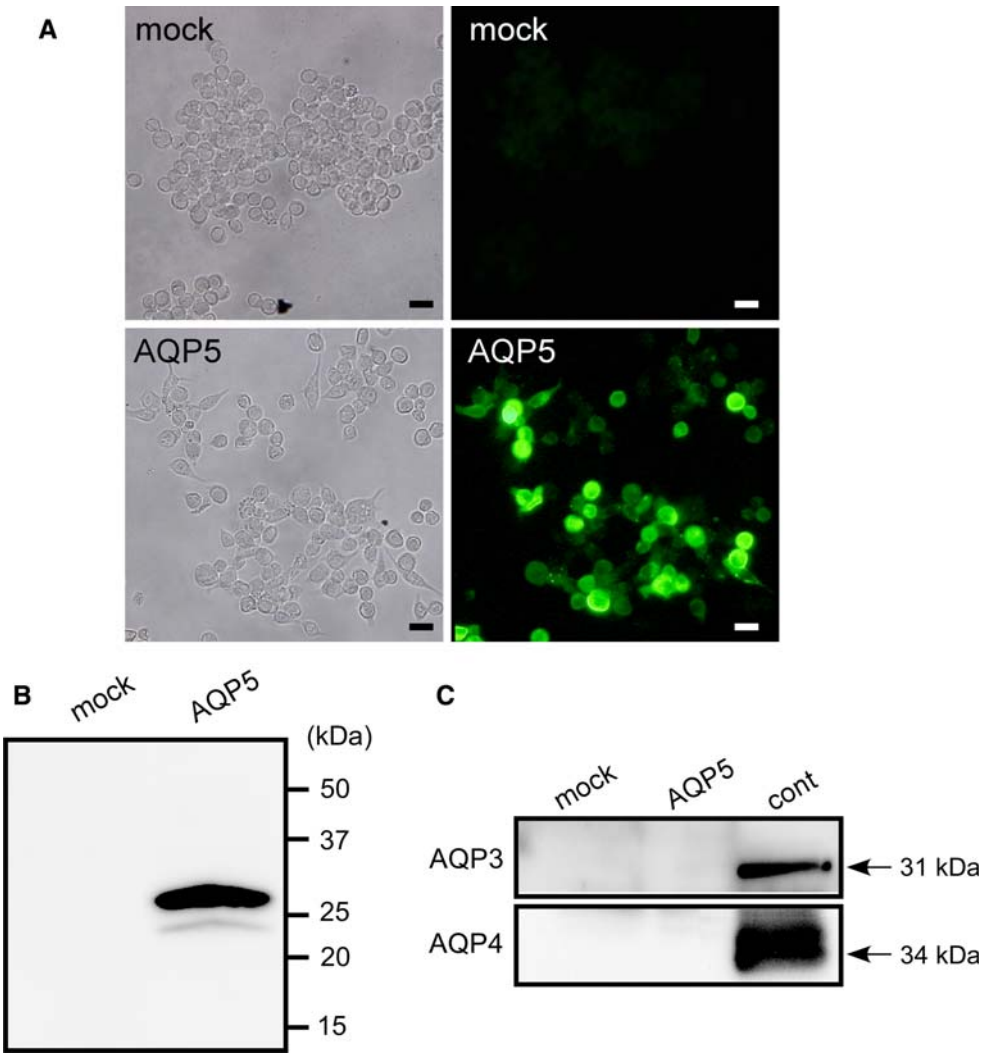

Fig. 3 Exogenous expression of AQP5 in MKN45, a poorly differentiated human gastric adenocarcinoma cell line. The cells were transfected with the AQP5-pcDNA4/His vector (AQP5) or pcDNA4/His vector (mock) at 24 h after seeding of the cells and cultured for 48 h. **a** Immunostaining for AQP5 in mock and AQP5-transfected cells. In *left panels*, optical images of cells are shown. In *right panels*, fluorescent images are shown. *Green color* indicates the expression of AQP5 protein in the cells. *Scale bars* 20 μm. **b** Western blotting for detecting AQP5 protein (27 kDa) in mock and the AQP5-transfected cells. **c** Western blotting for checking expression of AQP3 and AQP4 proteins in mock and the AQP5-transfected cells. Membrane fractions of rat kidney and human normal gastric mucosa (No. 5 in Table 1) were used as positive controls for AQP3 (31 kDa) and AQP4 (34 kDa), respectively

Next, we cultured the cells that were transfected with the AQP5 vector and found that the proportion of differentiated cells in the AQP5-transfected cell well was significantly greater than that in the mock cell well (Fig. 4c, d). Expression of AQP5 significantly increased the activity of alkaline phosphatase (Fig. 4e), which is known as a marker for the intestinal epithelial cell type of cancer cells [14]. Corresponding to the increased cell differentiation, the total cell number in the AQP5-transfected cell well was significantly smaller than that in the mock cell well (Fig. 4f).

It has been reported that laminin-5, heterotrimer of α3, β3 and γ2 chains, is continuously expressed along the tumor basement membrane in well differentiated gastric adenocarcinomas [15]. As shown in Fig. 5, we observed an increase in the expression level of laminin β3 in the AQP5-transfected MKN45 cells compared with mock cells.

AQP5-elicited water permeability is involved in the cell differentiation

Next, MKN45 cells that stably express AQP5 were constructed (Fig. 6a), and the effect of $HgCl_2$, an inhibitor of several AQPs including AQP5 [16, 17], on the cell differentiation was studied. In this experiment, the cells were treated with $HgCl_2$ intermittently twice for 10 min each at 24 and 48 h after seeding of the cells, and the effects were assessed at 72 h after seeding of the cells. It is noted that all of the cells showed round shape just after seeding of them. $HgCl_2$ (100 μM) decreased the proportion of differentiated cells (Fig. 6b) accompanying a decrease in the alkaline phosphatase activity in the cells (Fig. 6f). On the other hand, $HgCl_2$ increased the total cell number in the AQP5-expressing cell well (Fig. 6d). That is, the proportion of undifferentiated type of the AQP5-expressing cells increased in the presence of $HgCl_2$. In the MKN45 cells

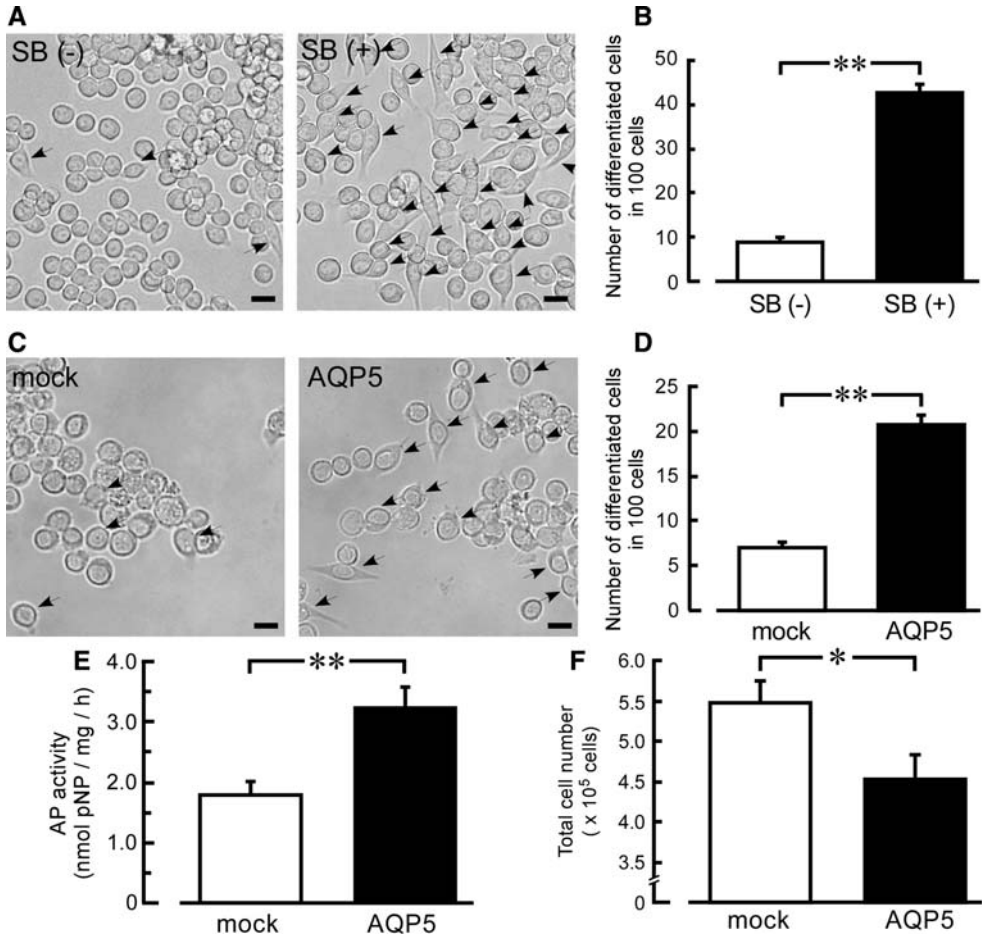

Fig. 4 Cancer cell differentiation induced by sodium butyrate or expression of AQP5. **a**, **b** MKN45 cells were cultured in the presence [SB(+)] and absence [SB(−)] of 2 mM sodium butyrate for 48 h. Sodium butyrate was added at 24 h after the cell seeding. In **a**, representative images are shown. Differentiated cells (spindle-shaped) are marked by *arrows*. In **b**, the number of differentiated cells in 100 cells in the presence and absence of SB are shown. $n = 20$. $^{**}p < 0.01$. **c**–**f** The cells were transfected with the AQP5-pcDNA4/His vector (AQP5) or pcDNA4/His vector (mock) at 24 h after the cell seeding and cultured furthermore for 48 h. In **c**, representative images are shown. In **d**, the number of differentiated cells in 100 cells transfected with mock and AQP5 are shown. $n = 20$. $^{**}p < 0.01$. In **e**, alkaline phosphatase (AP) activities in the AQP5-transfected cells and mock cells are shown. $n = 6$. $^{**}p < 0.01$. In **f**, the total cell numbers in the AQP5-transfected cell and mock cell wells are shown. The number of cells in a 12-well culture plate was assessed. In each well, 1×10^5 cells were seeded. $n = 6$. $^{*}p < 0.05$

that do not express AQP5 (control cells), $HgCl_2$ (100 μM) had no effects on the proportion of differentiated cells (Fig. 6c), the total cell number (Fig. 6e) or the alkaline phosphatase activity in the cells (Fig. 6g).

Discussion

The presence of AQP5 in the apical membrane of secretory glands has been reported in several normal digestive tracts [18–23]. On the other hand, the expression of AQP5 in human ovarian tissues was reported to be mainly localized in the basolateral membrane of benign tumor cells and in the apical and basolateral membranes of borderline cells, scattered in the membrane of malignant cells, and absent in the normal epithelium [24]. In human colorectal tissues, AQP5 mRNA was reported to be expressed in adenocarcinoma with almost no expression in surrounding normal mucosa [25]. Furthermore, pathophysiological functions of AQP5 protein were reported in colorectal [26] and non-small cell lung cancers [27]; that is, AQP5 acts as an oncogene in these cancers. These facts may indicate that the role of AQP5 in cancer cells is different from the role in normal secretory glands.

So far, the expression and function of AQP5 in human gastric cancer have not been clarified. In the present study, we have found that AQP5 is highly expressed in the intestinal type of adenocarcinoma. Up-regulated expression of AQP5 was localized in the apical membrane of the cancer cells. AQP5 was not expressed in intestinal metaplasia and the diffuse type of the cancer tissues. From pathophysiological aspects, the intestinal type is related to

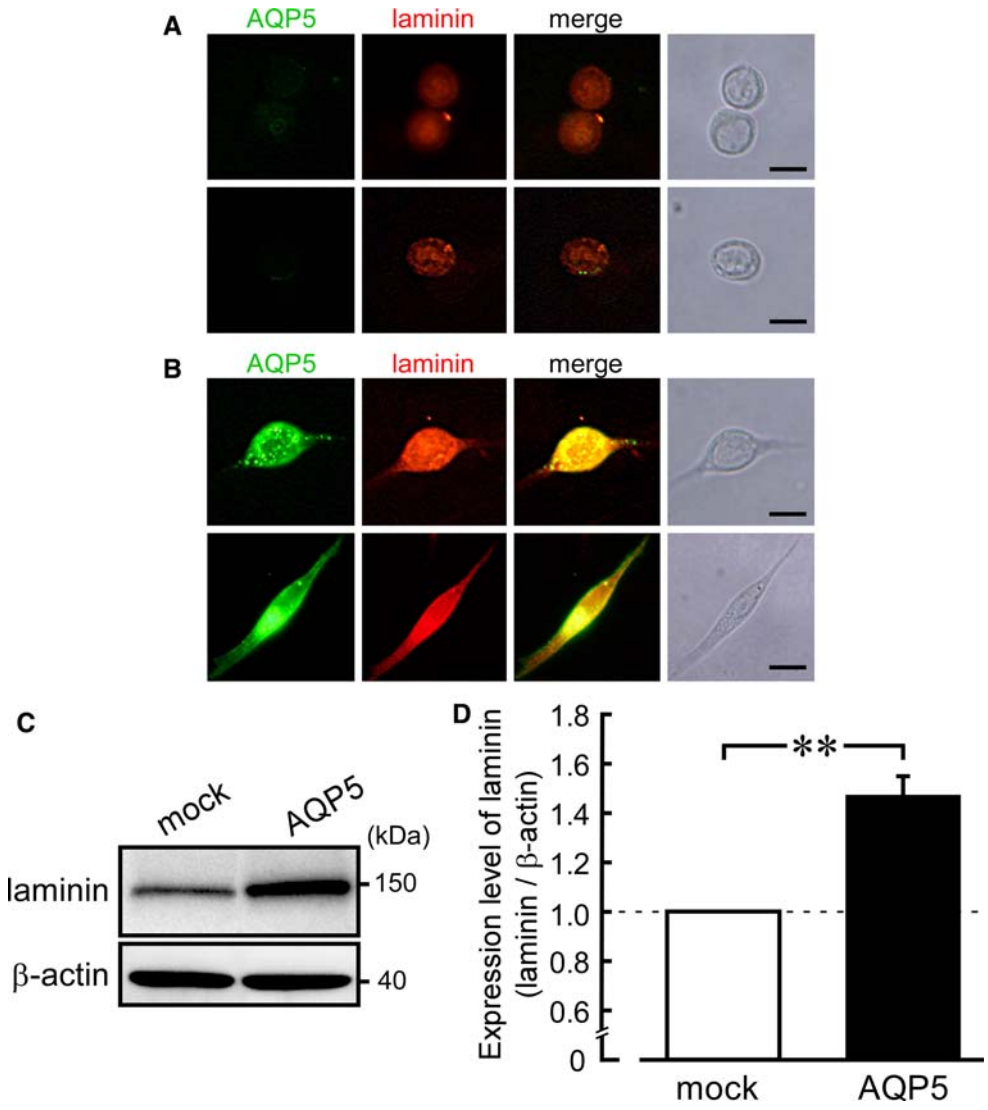

Fig. 5 Elevation of the expression level of laminin induced by expression of AQP5. The MKN45 cells were transfected with mock (**a**) or the AQP5 vector (**b**) at 24 h after seeding of the cells and followed by another 48 h culture. Double immunostaining was performed using anti-AQP5 (*green color*) and anti-laminin β3 (*red color*) antibodies. Localizations of AQP5 (*left side*), laminin β3 (*middle-left side*), and AQP5 plus laminin β3 (merged image; *middle-right side*) are shown. Optical images are shown in *right-side panels*. *Scale bar* 10 μm. **c** Western blotting of the membrane fraction from the AQP5-transfected cells or mock cells for laminin β3 and β-actin. **d** The level of protein expression of laminin β3 in the AQP5-transfected cells was compared with that of mock cells. The score for the normalized expression level of laminin β3 = [(amount of laminin β3 protein in the AQP5 cells)/(amount of β-actin protein in the AQP5 cells)]/[(amount of laminin β3 protein in mock cells)/(amount of β-actin protein in mock cells)]. The score for mock cells was normalized as 1. Averaged score ± SE of the AQP5-transfected cells are shown. $n = 6$. $^{**}p < 0.01$

corpus-dominant gastritis with gastric atrophy and intestinal metaplasia, whereas the diffuse type usually originates in pangastritis without atrophy [28]. The intestinal type is deeply related to infection with *Helicobacter pylori* [29]. Our above results suggest that AQP5 may be involved in the tumorigenesis pathway from intestinal metaplasia to intestinal type of gastric adenocarcinoma.

Interestingly, no significant expression of AQP4 was observed in the gastric cancer tissues, although AQP4 was abundantly expressed in the normal gastric mucosa. AQP4 was recently reported to relate to cell adhesion [30]. Therefore, the present results may indicate that the mechanism for maintaining polarity of the differentiated cancer cells may be different from that in normal gastric gland cells.

Exogenous expression of AQP5 in the poorly differentiated gastric adenocarcinoma cell line (MKN45) induced cell differentiation as judged from morphological (Fig. 4a–d) and functional aspects (Figs. 4e and 5). When the MKN45 cells in which AQP5 had been stably introduced were treated with $HgCl_2$, the proportion of differentiated cells were significantly decreased (Fig. 6b), whereas the total number of cells were increased (Fig. 6d). The treatment with $HgCl_2$ had no effects on the control MKN45 cells (Fig. 6c, e). Therefore, AQP5 may be associated with the mechanism of cancer cell differentiation, and the AQP5-increased water permeability may be involved in the differentiation of gastric cancer cells.

Our present results were opposite to previous studies in colorectal [26] and non-small cell lung cancers [27]. AQP5 induced cell proliferation via Ras/ERK/Rb pathway in colorectal cancer [26], and it promoted tumor invasion by interaction with c-Src in non-small cell lung cancer [27]. In both cases, phosphorylation on Ser156 of AQP5 is required. In a future study, we should clarify the down stream signaling pathway introduced by AQP5 in gastric cancer. It is also necessary to check whether there are copy number changes of AQP5 gene in the tumors.

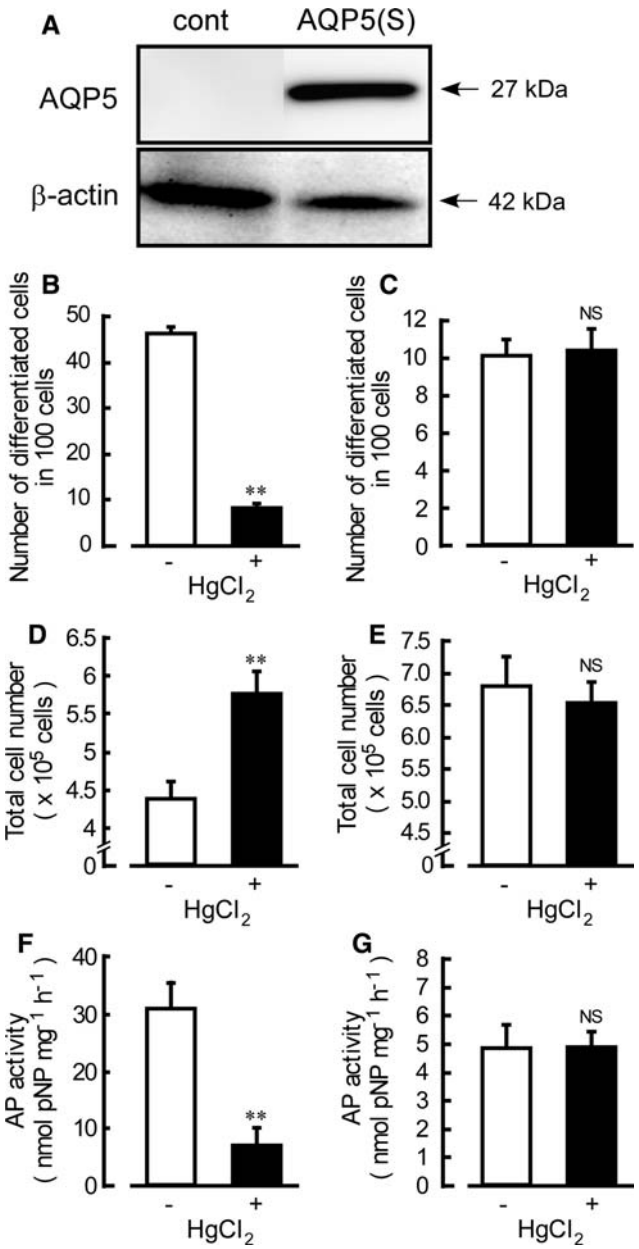

Fig. 6 $HgCl_2$-induced inhibition of the cell differentiation in the AQP5-expressing cells. **a** Western blotting for checking expression of AQP5 (27 kDa) in the mock cells (control cells; cont) and AQP5-expressing cells [stable expression of AQP5; AQP5(S)]. As a loading control, expression level of β-actin (42 kDa) was tested. **b**, **c** The number of differentiated cells in 100 cells stably expressing AQP5 (**b**) and in 100 control cells that do not express AQP5 (**c**). $n = 8$–10. **Significantly different versus zero $HgCl_2$ ($p < 0.01$). *NS* not significant versus zero $HgCl_2$ ($p > 0.05$). **d**, **e** The total cell number in the AQP5-expressing cell well (**d**) and control cell well (**e**). In each well, 1×10^5 cells were seeded. $n = 3$. $^{**}p < 0.01$; NS, $p > 0.05$. **f**, **g** Alkaline phosphatase (AP) activity in the AQP5-expressing cells (**f**) and control cells (**g**). $n = 3$. $^{**}p < 0.01$; NS, $p > 0.05$

Recently, Shimamura et al. [31] showed that transmembrane mucin MUC13 is up-regulated in the intestinal type of gastric cancer but not expressed in normal gastric mucosa. In the intestinal type, MUC13 was localized in the apical side of tubular gland lumen [31] as found in the localization of AQP5 (Fig. 2f). At present, the functional relationship between AQP5 and MUC13 in the gastric cancer is unknown and it would be an interesting subject to be clarified in future.

In conclusion, we have found that AQP5 is involved in the cancer cell differentiation of human gastric adenocarcinomas. For treatment of gastric cancer, modulation of AQP5 expression or function may be effective.

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