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P4H9-detected molecule expression on spindle-shaped fibroblasts indicates malignant phenotype of colorectal cancer

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Background: Our previous study using a mammary fat pad mouse model showed that P4H9, produced by the $\beta 2$ integrin epitope, detected a molecule on fibroblasts in response to carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)-expressing cancer cells. P4H9-detected molecule (PDM) expression appeared to be associated with myofibroblast differentiation. In this study, we investigated whether PDM is expressed on fibroblasts and cancer cells in clinical tissue samples, and whether the presence of PDM-expressing colorectal cancer cells is correlated with clinicopathological features of patients.

Methods: Immunohistochemistry was conducted to detect P4H9 on clinical tissue samples from 156 patients with colorectal cancer. Risk factors for metastases and survival were calculated for clinical implication of PDM-expressing spindle-shaped fibroblasts.

Results: Multivariate analysis showed that PDM-expressing spindle-shaped fibroblasts were an independent risk factor for lymph node metastasis, hematogenous metastasis, and poor survival. A Kaplan–Meier survival curve indicated that PDM-expressing spindle-shaped fibroblasts were associated with shorter survival time ($P<0.0001$). Immunofluorescence showed PDM expression on CCD-18Co fibroblasts and two colorectal cancer cell lines (HCT116 and HCT-15).

Conclusions: PDM-expressing spindle-shaped fibroblasts are associated with metastasis and shorter survival in colorectal cancer patients. PDM-expressing spindle-shaped fibroblasts may have a role in eliciting the malignant phenotype of colorectal cancer.

The concept that 'tumours are wounds that do not heal' was proposed by Dvorak (1986). Fibroblasts are mobilised during cancer progression. Fibroblasts in the colorectal cancer stroma, sometimes called activated fibroblasts, myofibroblasts, or tumour-associated fibroblasts, are the major stroma constituents of colorectal cancer tissues. Cancer-associated fibroblasts contribute to the invasion and metastatic process by inducing the epithelial–mesenchymal transition (EMT) of tumour cells (Kalluri, 2009; Thiery *et al*, 2009). Yeung *et al* (2013) reported that myofibroblasts are activated in colorectal cancer lymph node metastasis. Moreover, colorectal cancer myofibroblasts expressing α-smooth muscle actin (α-SMA) (Tsujino *et al*, 2007) or vimentin (Ngan *et al*, 2007) were associated with disease-free survival. Thus, to address the mechanism of cancer malignant phenotypes, it is important to investigate myofibroblast differentiation.

Our previous study using a mammary fat pad mouse model showed that phosphorylation mimic mutants of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)-4S (short cytoplasmic domain isoform) or wild-type CEACAM1-4L (long cytoplasmic domain isoform) induced myofibroblast differentiation in the surrounding breast stroma, which demonstrated elevated expression of the P4H9 $\beta 2$ integrin epitope on activated myofibroblasts (Yokoyama *et al*, 2007). These findings indicated that P4H9-detected molecule (PDM) may have a role in

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myofibroblast differentiation and thus may serve as a novel marker for this process.

P4H9 is produced by the epitope for β2 integrin. Integrins are α- and β-heterodimeric glycoproteins that are involved in cell–cell and cell–extracellular matrix interactions during embryogenesis, cell growth, apoptosis, immune reactions, wound healing, and tumour invasion and metastasis. β2 integrin is exclusively expressed on leukocytes. In general, the β1 and β3 integrins have an important role in cancer invasion and metastasis. Enhanced β1 integrin expression promotes metastatic potential (Chan *et al*, 1991), whereas the overexpression of β3 integrin is directly correlated with metastatic ability in melanoma (Cheresh, 1991). β2 integrin is also known as the β-subunit of LFA-1, Mac-1, and p150,95. The β-subunit was shown to be identical in all three proteins by physiochemical and immunochemical analyses (Sanchez-Madrid *et al*, 1983). This upregulation results in increased adhesiveness to endothelial cells and mediates localisation of leukocytes to inflammatory sites (Springer and Anderson, 1986). β2 integrin is a key factor in leukocyte adhesion.

The aim of the present study was to investigate whether PDM is expressed on fibroblasts and cancer cells in clinical tissue samples and, if so, whether PDM-expressing fibroblasts or cancer cells in colorectal cancer tissue are correlated with patients' clinicopathological features. Moreover, we examined PDM expression on normal colon fibroblasts and on colorectal cancer cell lines.

MATERIALS AND METHODS

Patients. A total of 156 patients with colorectal cancer who underwent surgery between January 2002 and November 2003 at Wakayama Medical University Hospital (WMUH) were enrolled in this study. Tumours were resected from either the colon ($n=83$) or the rectum ($n=73$). The patients included 28 stage-I, 63 stage-II, 36 stage-III, and 29 stage-IV colorectal carcinomas based on the TNM classification. The mean age of the patients was 62.3 years, and there were 93 male and 63 female subjects. The follow-up period was 10 years. Stage-III and -IV patients received 5-fluorouracil-based postoperative chemotherapy, whereas stage-I and -II patients received no chemotherapy. Of the 156 patients, 68 patients with colorectal cancer who underwent surgery were enrolled in a comparison study among PDM, vimentin, and α-SMA expression on fibroblasts between April 2002 and March 2003 at WMUH. The present study was approved by the Human Ethics Review Committee of WMUH.

Immunohistochemistry. Immunohistological staining using the streptavidin-biotin methods was performed. Tissue sections (4 μm thick) were prepared from formalin-fixed paraffin-embedded blocks. Sections were deparaffinised and autoclaved at 121 °C for 10 min in 0.1 M citrate buffer (pH 6.0). Endogenous peroxidase activities were blocked by incubation in 3% hydrogen peroxide for 5 min at room temperature. The slides were washed with PBS buffer solution and incubated with serum-free protein-blocking solution (X0909, Dako, Carpinteria, CA, USA) for 10 min at room temperature. Thereafter, the sections were incubated with the primary antibodies, mouse anti-human β2 integrin monoclonal antibodies (Santa Cruz Biotech, Dallas, TX, USA, P4H9; 1 : 200), mouse anti-human vimentin monoclonal antibodies (Dako; 1 : 200), or mouse anti-human α-SMA (Dako; 1 : 200) for 18 h at 4 °C. A biotinylated secondary antibody and peroxidase-conjugated streptavidin from the Histofine MAX-PO (M) kit (Nichirei, Tokyo, Japan) were applied for 30 min at room temperature. Finally, the sections were incubated in 3′3-diaminobenzidine for 10 min, followed by haematoxylin counterstaining and slide mounting. Stained leukocytes were used as internal control for immunohistochemistry by P4H9 (Supplementary Figure 1).

All specimens were blindly reviewed twice by three individuals including a pathologist (SY and JI). If discrepancies arose, the specimens were discussed to achieve a consensus while being viewed with a multihead microscope. The correlation between PDM expression and the clinicopathological characteristics of these patients was evaluated. All pictures were acquired using an Eclipse 80i (Nikon, Tokyo, Japan), with the NIS-Elements D 2.30 software program (Nikon).

Cell lines. Four human colorectal cancer cell lines were used in the present study. HT29, colo 201, HCT116, HCT15, and CCD-18Co cells were purchased from the American Type Culture Collection (Manassas, VA, USA). HT29 and HCT116 cells were maintained in McCoy's 5A (Gibco, Grand Island, NY, USA) medium containing 10% fetal bovine serum (FBS) (Gibco), 100 U ml^{-1} of penicillin G, and 100 μg ml^{-1} of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HCT-15 cells were maintained in Eagle's minimum essential medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS (Gibco), 100 U ml^{-1} of penicillin G, and 100 μg ml^{-1} of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Colo 201 cells were maintained in Roswell Park Memorial Institute 1640 Medium (Sigma-Aldrich) containing 10% FBS (Gibco), 100 U ml^{-1} of penicillin G, and 100 μg ml^{-1} of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. CCD-18Co cells were maintained in Eagle's minimum essential medium (Sigma-Aldrich) containing 10% FBS, 100 U ml^{-1} of penicillin G, and 100 μg ml^{-1} of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

Immunofluorescence microscopy. For the staining, the cells were grown on a film-bottomed dish (Matsunami Glass Inc., Osaka, Japan) and stained with mouse mAb (Santa Cruz Biotech, sc-13548, P4H9; 1 : 50) for 40 min followed by Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher Scientific Inc., Waltham, MA, USA, 1 : 100) and Hoechst33342 (Dojindo, Kumamoto, Japan) for 40 min. A Zeiss LSM700 laser scanning confocal microscope (Zeiss, Jena, Germany) was used to acquire data.

Statistical analysis. Each clinicopathological factor was separately assessed using the χ^2-test. The factors determined to be significant by the χ^2-test were analysed by a multivariate logistic regression, and an odds ratio with a 95% confidence interval (CI) was calculated for each factor. The Kaplan–Meier method was used to estimate the survival of colorectal cancer patients, and the log-rank test was used to determine the statistical significance. A Cox proportional hazards model was used to assess the risk ratio under simultaneous contributions from several covariates. The associations between discrete variables were assessed using the χ^2-test. All data are expressed as the mean ± s.d. A P-value of less than 0.05 was considered to be statistically significant. Statistical calculations were performed using the StatView ver. 5.0 software program (SAS Institute, Cary, NC, USA).

RESULTS

Association among PDM, vimentin, and α-SMA expression on fibroblasts and cancer cells. Sixty-eight patients with colorectal cancer who underwent surgery between April 2002 and March 2003 at WMUH were enrolled in a comparison study of PDM, vimentin, and α-SMA expression on fibroblasts. PDM expression on fibroblasts was associated with α-SMA expression, but not with vimentin expression. PDM expression on cancer cells was correlated with PDM expression on fibroblasts, but not with vimentin and α-SMA expression on fibroblasts (Table 1).

P4H9-detected molecule expression by spindle-shaped fibroblasts and metastases. In clinical tissues, colorectal cancer cells and fibroblasts expressed PDM. There were two types of PDM-expressing fibroblasts, rounded or spindle shaped (Figure 1).

Table 1. Association among PDM, vimentin, and α-SMA expression on fibroblasts and cancer cells

	PDMSF			PDM on cancer cells		
	Present	**Absent**	***P*-value**	**Present**	**Absent**	***P*-value**
Vimentin on FBs			0.151			0.574
Present	23	37		39	21	
Absent	1	7		6	2	
α-SMA on FBs			0.003			0.459
Present	23	28		35	7	
Absent	1	16		10	16	
PDMSF						0.004
Present	—	—		16	8	
Absent	—	—		29	15	

Abbreviations: FB = fibroblast; PDM = P4H9-detected molecule; PDMSF = P4H9-detected molecule expression on a spindle-shaped fibroblast; α-SMA = α-smooth muscle actin.

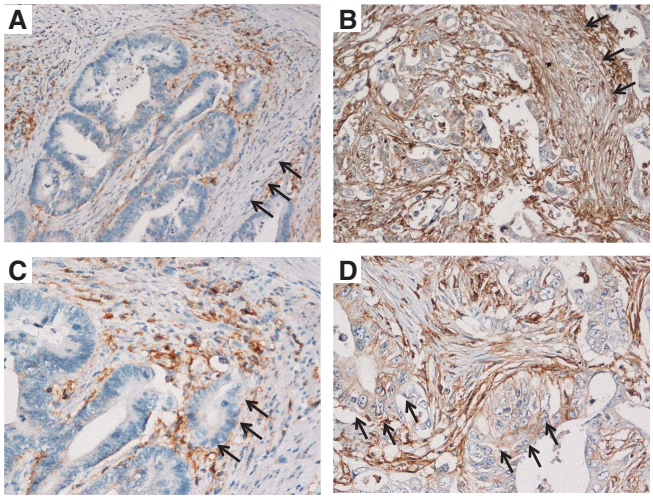

Figure 1. Immunohistochemical analyses for P4H9-detected molecule (PDM) on clinical samples. (**A**) Less expression of PDM on spindle-shaped fibroblasts (arrows, ×200). (**B**) Expression of PDM on spindle-shaped fibroblasts (arrows, ×200). (**C**) Less expression of PDM on cancer cells with less expression of PDM on spindle-shaped fibroblasts (arrows, ×400). (**D**) Expression of PDM on cancer cells with expression of PDM on spindle-shaped fibroblasts (arrows, ×400).

On the basis of the PDM expression and shape of fibroblasts, patients were divided into two groups: patients with PDM-expressing spindle-shaped fibroblasts ($n = 54$) and patients without PDM-expressing spindle-shaped fibroblasts ($n = 102$).

To investigate whether the existence of PDM-expressing spindle-shaped fibroblasts is correlated with lymph node involvement and hematogenous metastases, statistical analyses were performed between the presence of PDM-expressing spindle-shaped fibroblasts and clinicopathological characteristics. A χ^2-test for lymph node metastasis revealed that PDM-expressing spindle-shaped fibroblasts, differentiation, depth of tumour invasion, lymphatic permeation, and venous permeation were statistically correlated (Table 2). A multivariate logistic regression analysis for lymph node metastasis revealed PDM-expressing spindle-shaped fibroblasts to be an independent risk factor (odds ratio = 7.387; 95% CI = 1.338–6.030; *P*-value = 0.0066). A χ^2-test for hematogenous metastasis revealed that PDM-expressing spindle-shaped fibroblasts, differentiation, depth of tumour invasion, lymphatic permeation, and venous permeation were statistically correlated (Table 2). Likewise, the only independent risk factor in a multivariate logistic regression analysis for lymph node and hematogenous metastasis was PDM-expressing spindle-shaped fibroblasts (lymph node metastasis: odds ratio = 7.387; 95% CI = 1.338–6.030; *P*-value = 0.0066; hematogenous metastasis: odds ratio = 7.358; 95% CI = 2.532–21.389; *P*-value = 0.0002). These results suggested that the presence of PDM-expressing spindle-shaped fibroblasts is associated with invasion and metastases of colorectal cancer.

P4H9-detected molecule expression by spindle-shaped fibroblasts and survival. To address whether PDM-expressing spindle-shaped fibroblasts are associated with shorter survival of patients with colorectal cancer, we statistically analysed the histological findings of surgically resected primary lesions related to survival of colorectal cancer patients. The univariate analyses for survival are summarised in Table 3. Differentiation, depth of the tumour invasion, lymphatic permeation, venous permeation, lymph node metastasis, hematogenous metastasis, and PDM-expressing spindle-shaped fibroblasts were found to have prognostic value. A multivariate analysis of those parameters with significant prognostic values in the univariate analysis revealed that lymph node metastasis and PDM-expressing spindle-shaped fibroblasts were independent prognostic factors (Table 3). The Kaplan–Meier analysis for overall survival showed that the presence of PDM-expressing spindle-shaped fibroblasts was significantly associated with a shorter survival time ($P < 0.0001$; Figure 2). These results indicated that PDM-expressing spindle-shaped fibroblasts are associated with survival of colorectal cancer patients.

Expression of PDM on fibroblasts and colorectal cancer cell lines. Immunofluorescence without permeabilisation demonstrated that PDM was expressed on the cell surface of HCT116 and HCT-15 colon cancer cell lines and CCD-18Co rectal fibroblast cell line (Figure 3), whereas HT29 and colo 201 cells do not express PDM.

DISCUSSION

PDM expression by fibroblasts and cancer cells was observed in clinical colorectal cancer tissues in the present study. Only PDM expression on fibroblasts was associated with cancer metastases and poor survival. PDM was significantly associated with α-SMA, indicating that PDM may be related to myofibroblast differentiation. Immunofluorescence without permeabilisation showed that PDM was expressed on the cell surface of these cells, indicating that PDM differs from vimentin and α-SMA, which exist inside the cell. Thus, PDM may serve as a novel fibroblast marker.

PDM expression on cancer cells was significantly associated with PDM expression on fibroblasts, suggesting that PDM may

Table 2. Univariate analyses of lymph node metastasis and hematogenous metastasis

	Lymph node metastasis			Hematogenous metastasis		
	Present	**Absent**	***P*-value**	**Present**	**Absent**	***P*-value**
Gender			0.065			0.33
Male	32	61		19	76	
Female	31	32		9	54	
Tumour site			0.54			0.073
Colon	35	47		19	63	
Rectum	28	46		9	65	
Differentiation			0.0019			0.0038
Well	24	15		15	102	
Others	39	78		13	26	
Depth			0.0073			0.0063
T3,4	58	70		28	28	
T1,2	5	23		0	100	
Lymphatic permeation			0.0035			0.027
Present	56	64		26	94	
Absent	7	29		2	34	
Venous permeation			0.0015			0.0003
Present	54	58		28	44	
Absent	9	35		0	84	
PDM cancer cells			0.89			0.47
Present	44	64		21	87	
Absent	19	29		7	41	
PDMSF			<0.0001			<0.0001
Present	34	20		22	32	
Absent	29	73		6	96	

Abbreviations: PDM = P4H9-detected molecule; PDMSF = P4H9-detected molecule expression on a spindle-shaped fibroblast. Well refers to well-differentiated adenocarcinoma; PDM cancer cells, P4H9-detected molecule expression on cancer cells.

Table 3. Univariate and multivariate analyses of histological factors related to survival

	Univariate analysis			Multivariable analysis		
Variable	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Differentiation (others)	3.0	1.365–6.611	0.0063			
Depth (T3, T4)	5.1	1.148–22.551	0.032			
Lymphatic permeation	15.602	2.059–118.249	0.0078			
Venous permeation	4.359	1.445–13.148	0.009			
Lymph node metastasis	5.591	2.504–12.483	<0.0001	3.17	1.323–7.618	0.0097
PDM cancer cells	1.051	0.478–2.313	0.901			
PDMSF	5.901	2.677–13.011	<0.0001	3.09	1.295–7.387	0.011

Abbreviations: CI = confidential interval; HR = hazard ratio; PDM = P4H9-detected molecule; PDMSF = P4H9-detected molecule expression on a spindle-shaped fibroblast. Others, not well-differentiated adenocarcinoma; PDM cancer cells, P4H9-detected molecule expression on cancer cells.

have a role in the interaction between cancer cells and fibroblasts. Immunofluorescence for cancer cell lines showed that HCT116, a spindle-shaped cell, expressed PDM, whereas PDM expression on HT29, which is not spindle shaped, was faint. HCT-15, adherent cells revealing spread on plastic, expressed PDM, whereas colo 201 cells, suspension cell with some loosely adherent cells, expressed faint PDM. These findings suggest that PDM may be associated with cell morphology, such as the EMT. Further research is required to address the relationship between PDM expression and cell morphology.

PDM expression on spindle-shaped fibroblasts was significantly associated with the malignant phenotype, including metastasis and survival. It has been reported that the desmoplastic reaction of fibrotic cancer stroma is an independent prognostic index for resectable colorectal liver metastasis (Ueno *et al*, 2014). Thus, a combination of histological change and PDM expression on fibroblasts may be a novel myofibroblast-differentiation marker for the malignant phenotype of colorectal cancer.

Our previous study revealed that P4H9, produced by the epitope for $\beta 2$ integrin, detected a molecule on fibroblasts induced by CEACAM1 expression on cancer cells in a mammary fat pad mouse model; PDM expression was associated with myofibroblast differentiation (Yokoyama *et al*, 2007). CEACAM1 may be an inducer of PDM on fibroblasts. CEACAM1 is downregulated in colorectal adenoma (Nollau *et al*, 1997) and during the early stage of colorectal cancer (Neumaier *et al*, 1993). Previous investigations, including our study, showed that at more advanced stages CEACAM1 was re-expressed at the invasive front of advanced colorectal cancer, and its expression level was correlated with the clinical stage (Jantscheff *et al*, 2003; Kang *et al*, 2007; Ieda *et al*,

2011). The dominant isoform of CEACAM1 with a long cytoplasmic domain (*vs* the short CEACAM1 isoform) at the invasion front of colorectal cancer is associated with metastases to lymph node and distant sites and poor survival of patients (Ieda *et al*, 2011). Colorectal cancer cells expressing the dominant CEACAM1 long cytoplasmic domain isoform form a solid spheroid beyond the invasion front, and then the spheroid becomes a hollow spheroid via inner cell apoptosis (Kirshner *et al*, 2003; Yokoyama *et al*, 2007; Tamura *et al*, 2011). Hollow spheroid formation beyond the invasive front of colorectal cancer is correlated with lymph node and distant metastasis and shorter survival (Tamura *et al*, 2011). CEACAM1 long cytoplasmic domain dominance and hollow spheroid formation was shown to be a colorectal phenotype of chemoresistance to 5-fluorouracil (Yamamoto *et al*, 2015). Taken together, these findings indicate that CEACAM1 is associated with the colorectal cancer malignant phenotype. The current study revealed the significance of PDM expression by spindle-shaped fibroblasts in colorectal cancer tissues. Thus, investigation of the interaction between cancer cells expressing CEACAM1 and myofibroblasts expressing PDM is warranted.

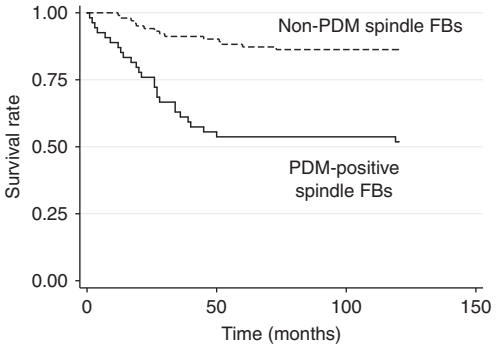

Figure 2. Kaplan–Meier plots of the overall patient survival. The 10-year survival rates of patients with P4H9-detected molecule-positive spindle-shaped fibroblasts (PDM-positive spindle FBs) and patients without PDM-positive spindle-shaped fibroblasts (non-PDM spindle FBs) were analysed.

In this and our previous study (Yokoyama *et al*, 2007), P4H9 was shown to detect a molecule on cancer cells. We also verified that other cancer cells, including human breast cancer cell line MCF7 and human prostate cancer cell line PC-3, express PDM. The most important task is to elucidate what P4H9 detects. Unfortunately, P4H9 is not applicable for western blot analysis at this moment. Moreover, CCD-18Co fibroblasts reveal limited passage. Further cellular and molecular analyses are needed to address what P4H9 detects.

In general, $\beta 2$ integrin is expressed only on leukocytes, but the present study showed that cancer cells also expressed PDM. Clinical data suggested that the molecule detected by P4H9 is associated with the malignant phenotype of colorectal cancer, and preliminary basic research has shown that PDM may be related to the EMT. PDM is not consistent with other fibroblast-related molecules, such as vimentin and α-SMA. It might be a $\beta 2$-integrin-like molecule or a novel fibroblast or EMT marker. Further molecular analyses such as proteomics including western blot

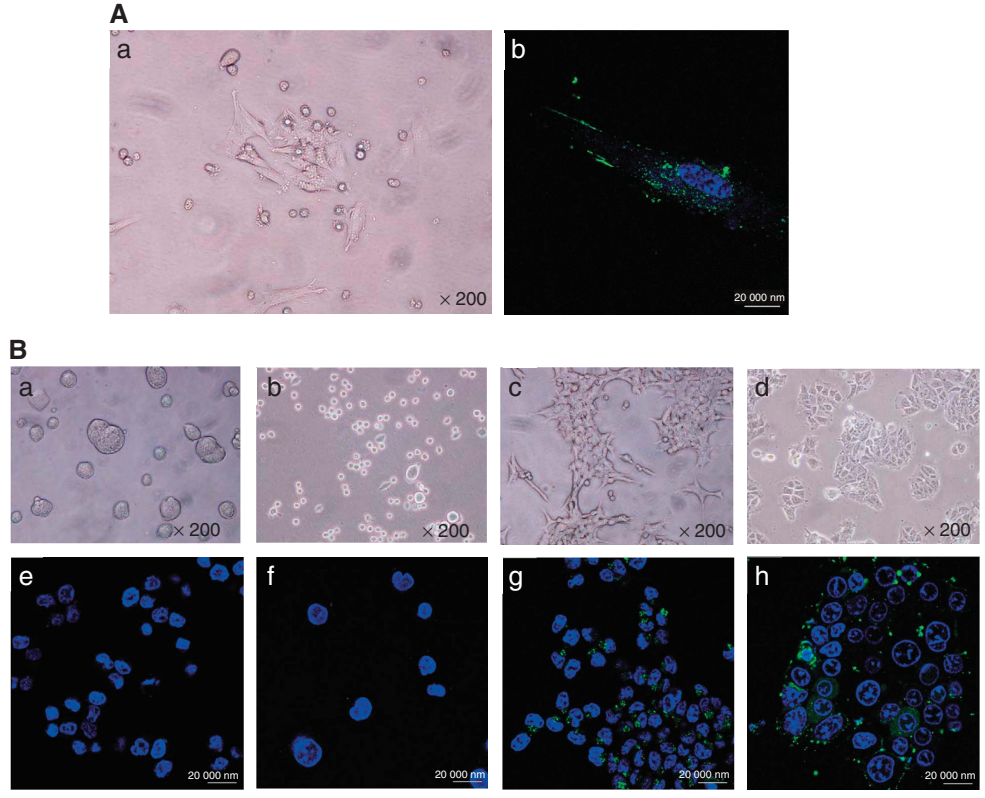

Figure 3. Morphology of colorectal cancer cells and fibroblasts and P4H9-detected molecule expression. (**A**: a) Morphology of fibroblasts observed by inverted microscopy (× 200). (**A**: b) Immunofluorescence with P4H9 for fibroblasts and colorectal cancer cell lines (green: P4H9; blue: Hoechst 33342). (**B**: a–d) Morphology of colorectal cancer cells observed by inverted microscopy (× 200), (**B**: e–h) Immunofluorescence with P4H9 for colorectal cancer cell lines (green: P4H9; blue: Hoechst 33342). (**A**: a and b) CCD-18Co; (**B**: a and e) HT29; (**B**: b and f) colo 201; (**B**: c and g) HCT116; (**B**: d and h) HCT-15.

analysis and genome bioinformatics in a larger unbiased database will be needed to identify the molecule.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)