〈Regular Article〉

Role of the expression of collagen prolyl-4-hydroxylase α subunits 1 and 2 in the development and prognosis of breast cancer

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ABSTRACT **Background**: The expression of prolyl-4-hydroxylase (P4H), an enzyme involved in collagen biosynthesis, is significantly upregulated during breast cancer development and progression. However, the molecular mechanisms by which P4H expression in cancer cells induces progression have not been elucidated. Thus, we aimed to determine the significance of the expression of isoforms 1 and 2 of P4H in breast cancer.

Methods: We performed immunohistochemical analysis for P4HA1 and P4HA2 on the tumor samples obtained from 182 patients with breast cancer and examined the correlation between clinicopathological factors and markers related to epithelial-mesenchymal transition and ischemia. Protein expression levels were investigated using western blotting. In addition, breast cancer cell cultures were used to characterize the expression.

Results: Expression of both P4HA1 and P4HA2 was upregulated in cancer cells compared with that in normal mammary glands; the high-P4H expression group tended to have a poorer prognosis than the low-P4H expression group. In particular, P4HA2 was strongly associated with tumor grade; P4HA2 expression showed a weak negative correlation with HIF-2α expression. In cultured breast cancer cells, the immunohistological expression of P4H and HIF increased to various degrees under hypoxia, while P4H protein levels increased in a time-dependent manner.

Conclusion: P4HA2 can be used as a marker of breast cancer grade and a prognostic factor. Differential expression of P4HA1 and P4HA2 was observed in an ischemic environment, suggesting that each may be affected by the type of collagen involved.

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Key words：Breast cancer, Prolyl-4-hydroxylase (P4H), Triple-negative breast cancer (TNBC), Hypoxia inducible factor (HIF), Epithelial-mesenchymal transition (EMT), Immunohistochemistry

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INTRODUCTION

Breast cancer is one of the most common cancers in women and its incidence is increasing[1]. However, the cause of breast cancer has not been elucidated, and there are no definitive measures to prevent its occurrence. Therefore, early detection and treatment through medical checkups are important.

The histopathological forms of breast cancer vary, and there are more than 30 different histological types of invasive cancers, including rare cancers. In routine pathological diagnosis, the grade (degree of atypia) and biomarker expression status are reported, and the latter includes hormone receptors (ER: estrogen receptor, PR: progesterone receptor), human epidermal growth factor receptor 2 (HER2), and Ki67, which are examined by immunohistochemistry. Surrogated intrinsic subtyping (luminal A, luminal B, HER2, and triple-negative) based on the combination of these four markers is important for drug selection and prediction of therapeutic efficacy and prognosis[2-4].

Borderline malignant lesions, such as atypical ductal hyperplasia (ADH), have been indicated as significant precancerous lesions in a limited number of low-grade hormone receptor-positive breast cancers. In contrast to hormone-dependent breast cancers, such as luminal A and luminal B, hormone-independent breast cancer, especially triple-negative breast cancer (TNBC), is well known as an aggressive histological subtype with poor prognosis and a high rate of relapse or metastasis[5-7]. Additionally, the mechanism underlying the outbreak, a precursor of TNBC, is not completely understood. Considering the ER-negative, PR-negative, and HER2-negative status of TNBC, chemotherapy regimens are regarded as gold standard treatments for TNBC; however, more than 50% of patients are likely to experience cancer recurrence in the first 3-5 years after treatment[8]. Therefore, it is important to detect a precursor or new prognostic factor(s) that can serve as a new therapeutic target for TNBC.

Collagen is the main component of the extracellular matrix (ECM), which forms the local microenvironment of cancer cells with fibroblasts, immunocytes, and endothelial cells. Abnormal collagen deposition in the ECM may supply a variety of cytokines and growth factors, such as transforming growth factor (TGF)-β, hepatocyte growth factor (HGF), and epidermal growth factor (EGF), thereby causing the cancer cells to undergo epithelial-mesenchymal transition (EMT), further resulting in invasion and metastasis[9-13].

Prolyl-4-hydroxylase (P4H) is a common post-translational modifying enzyme in collagen biosynthesis, which plays an important role in collagen triple helix formation and stabilization[14]. P4H is an $\alpha_2\beta_2$ tetrameric α-ketoglutarate (α-KG)-dependent dioxygenase that catalyzes the 4-hydroxylation of proline. The catalytic activation of these reactions requires a greater quantity of the P4H α subunit (P4HA), whose three isoforms (P4HA1, P4HA2, and P4HA3) have been identified in mammalian cells[15]. P4HA1 is the major isoform in most cells and tissues, and P4HA2 exists in osteoblasts, chondrocytes, and endothelial cells. P4HA3 was observed in most cells but at low levels. P4H expression, mainly P4HA1 and P4HA2, has been detected in many solid tumors, including oral cavity squamous cell carcinoma[16], pancreatic carcinoma[17], and invasive breast carcinoma[18]. The expression of P4H is significantly upregulated during breast cancer development and progression[19, 20]. Interestingly, a study suggested that P4HA1 is associated with hypoxia inducible factor (HIF)-1α stabilization and TNBC chemoresistance[21]. However, P4H subtypes and their association with TNBC and the molecular mechanisms by which P4H expression in cancer cells induces progression remain unclear and were the subject of our study.

In this study, we aimed to clarify the significance of P4H expression in breast cancers by focusing on P4H expression and comparing it with clinicopathological factors and the expression of other biomarkers, such as HIF-1α, HIF-2α, and EMT transcription factors, Twist and zinc finger E-box binding homeobox 1 (ZEB1).

MATERIALS AND METHODS

Patients

This study was approved by the Ethics Committee of Kawasaki Medical School and Kawasaki Medical School Hospital (Study No. 3584-03). This study was conducted in compliance with the principles of the Declaration of Helsinki. Blanket consent to use pathology materials for research was obtained from the patients who were given the opportunity to refuse participation, and information pertaining to this study was disclosed on the website of the Kawasaki Medical School Hospital. Patients diagnosed with primary breast cancer who underwent surgery at Kawasaki Medical School Hospital between January 2007 and December 2020 were enrolled in this study. Among these patients, 182 patients diagnosed with primary invasive breast cancer with tumor diameter ≥ 10 mm were included. None of the patients were treated with preoperative therapy. Patients for whom informed consent was not obtained, cases of recurrence, and those with insufficient demographic, clinical, and pathological data were excluded. Overall survival (OS) and recurrence-free survival (RFS) were the primary outcomes of this study. The histological subtype and/or grading was assigned according to the 5th edition of the WHO classification and the 8th edition of the TNM classification established by the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC).

Human breast cancer tissue samples

In all the cases, a portion of the tumor tissue obtained during surgery was quickly frozen in liquid nitrogen and stored at -80℃ until protein extraction. The remaining tumor tissue sample was fixed in 10% neutral buffered formalin and embedded in paraffin wax.

Breast cancer cell lines and cell culture

Human breast cancer cell lines MCF-7, KPL-4, MDA-MB-157, and MDA-MB-231 were used in this study. MCF-7, MDA-MB-157, and MDA-MB-231 cell lines were obtained from Dr. Robert Dickson (Lombardi Cancer Research Center, Washington, DC, USA). KPL-4 was established at the Department of Breast and Thyroid Surgery of Kawasaki Medical School and its biological characteristics have been described previously[22]. MCF-7 cells expressed a high level of ER but no detectable HER2; KPL-4 cells expressed a high level of HER2 but no detectable ER; MDA-MB-157 and MDA-MB-231 cells expressed neither ER nor HER2. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, US) containing 10% fetal bovine serum (FBS), 0.02% kanamycin for 3 or 4 days under normoxia (5% CO_2) or hypoxia (1% O_2) at 37℃.

Cell blocks

After culturing under normoxia or hypoxia, the breast cancer cells were washed with phosphate-buffered saline (PBS). Next, 0.25% trypsin, and 0.2% EDTA per 100 mm^2 of culture dish were added to detach the cells. The cells were then incubated for 7-10 min in a CO_2 incubator at 37℃. Then, 7.5 ml of DMEM containing 10% FBS was added to the culture dish to deactivate the trypsin, and the detached cells were transferred to 15 ml tubes. The cells were then centrifuged at 1200 rpm for 5 min to form a pellet. The supernatant was removed, the pellets washed with PBS, and the cells centrifuged at 1200 rpm for 5 min. After removing

the supernatant, 500 μl of 10% neutral buffered formalin was added to the cell pellets and mixed well. Finally, the formalin-fixed cell clots were embedded in paraffin wax.

Immunocytochemistry and immunohistochemistry

All tissue specimens used for pathological diagnosis of 182 breast cancer cases were reviewed following surgery, and one formalin-fixed paraffin-embedded specimen was extracted per case from the area of high tumor invasion. Unstained specimens were prepared from paraffin blocks for immunohistochemical staining. Accordingly, tissue sections of 4 μm thickness were dewaxed and hydrated. Antigen retrieval was performed in a hot water bath of Tris-EDTA buffer solution (pH 9.0) at 98℃ for 40 min. The sections were incubated with 3% H_2O_2 for 5 min at room temperature to block endogenous peroxidase activity. They were then washed with Tris-buffered saline (TBS) and incubated with the following primary antibodies for 30 min at room temperature or overnight at 4℃: rabbit polyclonal P4HA1 antibody (12658-1-AP, Proteintech, dilution 1:400), mouse monoclonal P4HA2 antibody (CL0351, ab211527, Abcam, dilution 1:50), rabbit monoclonal HIF-1α antibody (EP1215Y, ab51608, Abcam, dilution 1:100), mouse monoclonal HIF-2α antibody (ep190b, ab8365, Abcam, dilution 1:100), mouse monoclonal p53 antibody (DO-7, M7001, Dako, dilution 1:50), mouse monoclonal Twist antibody (Twist2c1a, ab50887, Abcam, dilution 1:400), and mouse monoclonal ZEB1 antibody (H-3, sc-515797, Santa Cruz Biotechnology, dilution 1:200). Next, the sections were washed in TBS, and color was developed using the EnVision Plus System or CSA Ⅱ System (Dako), according to the manufacturer's instructions; the sections were then counterstained with hematoxylin. Negative controls were treated in the same manner but without primary antibodies.

Leica Bond™ automated immunohistochemistry (IHC) and in situ hybridization system (BOND-III, Leica Microsystems) were used for the detection of ER, PR, and Ki67, using mouse monoclonal ER antibody (6F11, NCL-L-ER-6F11, Leica, dilution 1:200), mouse monoclonal PR antibody (16, NCL-PRG-312, Leica, dilution 1:1000), and mouse monoclonal Ki67 antibody (MIB-1, M7240, Dako, dilution 1:100), respectively. HER2 was also stained on the BOND-III stainer using the BOND Oracle HER2 IHC System (CB11, TA9145, Leica) according to the recommended protocol.

All immunostained slides were independently reviewed and evaluated by two pathologists (Y. M. and T.M.). ER and PR expression with nuclear staining in more than 1% of the tumor cells was considered positive. HER2 expression was evaluated according to the criteria of HercepTest scoring[23]. Ki67 scoring was carried out by counting at least 500 malignant invasive tumor cells in high-power fields, regardless of the staining intensity, and calculated the positive rate.

Histoscore (H-score) was evaluated semi-quantitatively based on staining intensity as score 0 (negative), score 1 (weakly positive), score 2 (moderately positive), and score 3 (strongly positive), and the percentage of stained cells (0–100%), including nuclear and/or cytoplasmic staining[24]. H-Score was calculated as follows: H-score = [0 × (% cells with 0 intensity)] + [1 × (% cells with 1 intensity)] + [2 × (% cells with 2 intensity)] + [3 × (% cells with 3 intensity)]. P4HA1 and P4HA2 were evaluated based on the intensity of cytoplasmic staining, whereas HIF-1α, HIF-2α, Twist, and ZEB1 were evaluated based on the intensity of nuclear staining (Fig. 1). The cutoff values of each marker were defined as the median values of their H-scores for the respective immunostaining. Regarding p53 staining, cases with nuclear staining in more than 10% of the tumor cells were considered positive.

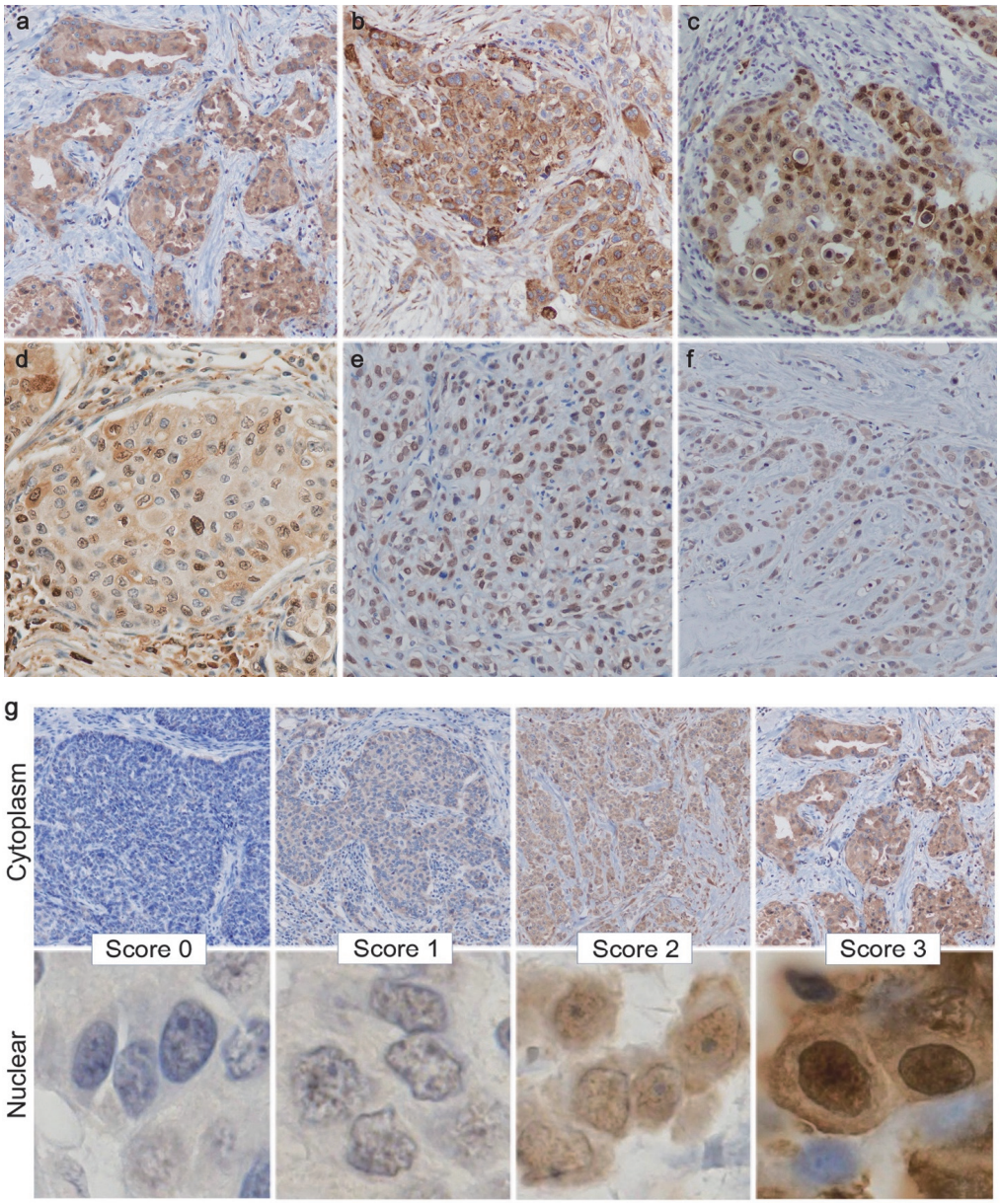

Fig. 1. Immunohistochemical staining of P4HA1 (a), P4HA2 (b), HIF-1α (c), HIF-2α (d), Twist (e), and ZEB-1 (f) in human breast cancer tissue samples. Histoscore was evaluated based on staining intensity using four scores (g). P4HA1 and P4HA2 were evaluated using the cytoplasmic scores in the upper row. HIF-1α, HIF-2α, Twist, and ZEB-1 were evaluated using the nuclear scores in the lower row.

Western blot assays

Breast cancer cells were washed with PBS and lysed for protein extraction using Pierce™ RIPA Buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific). The breast cancer tissue samples were lysed in cell lysis buffer (Cell Signaling Technology) containing 1% protease inhibitor mixture (Sigma-Aldrich) and 100 mmol/L phenylmethylsulphonyl fluoride. Total protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

All proteins were resolved by 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to a nitrocellulose membrane (Thermo Fisher Scientific). The membranes were blocked with blocking buffer containing 5% BSA and 0.2% Tween 20 in TBS at room temperature for 1 h and subsequently incubated overnight at 4℃ with the following primary antibodies: rabbit polyclonal P4HA1 antibody (12658-1-AP, Proteintech, dilution 1:2000), mouse monoclonal P4HA2 antibody (CL0351, ab211527, Abcam, dilution 1:1000), rabbit monoclonal HIF-1α antibody (P1215Y, ab51608, Abcam, dilution 1:1000), and mouse monoclonal HIF-2α antibody (ep190b, ab8365, Abcam, dilution 1:1000). The samples were incubated with secondary antibodies, goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, dilution 1:10000) or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, dilution 1:10000), at room temperature for 1 h. Protein bands were visualized using SuperSignal™ West Pico Plus chemiluminescent substrate (Thermo Fisher Scientific) using an Amersham Imager 680 (GE Healthcare). β-actin (Sigma-Aldrich, dilution 1:10000) as a loading control.

Statistical analysis

Analysis was performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)[25]. More precisely, it is a modified version of the R commander designed to add statistical functions frequently used in biostatistics.

The association between P4HA1 and/or P4HA2 expression and clinicopathological parameters was assessed using the Chi-square test or Fisher's exact test. The Mann–Whitney U test was used when the data did not follow a normal distribution or when ordinal variables were compared. One-way analysis of variance (ANOVA) was used to analyze the continuous variables. Repeated measures ANOVA was used to compare the expression levels of various proteins over time. Spearman's correlation test was used to examine the relationship between the expression of the respective biomarkers. OS and RFS were assessed using Kaplan-Meier curves and log-rank tests. Univariate and multivariate analyses were performed using Cox regression analysis. All tests were two-tailed, and $p < 0.05$ was considered as statistically significant.

RESULTS

Patients and association between P4H expression and clinicopathological parameters

Among the 182 patients, the mean age was 62.11 ± 14.86 years (range 25-91). There were 99 patients with non-triple-negative (TN) type (51 cases of luminal A type and 48 cases of HER2-enriched type) and 83 with TN type. The median follow-up period was 59.7 months.

First, we compared the expression of P4HA1 and P4HA2 in non-neoplastic ductal epithelium and breast cancer cells (Fig. 2). In 146 cases of P4HA1 and 150 cases of P4HA2, a non-neoplastic mammary epithelium was found in the same specimen. We found that both P4HA1 and P4HA2 were upregulated in breast cancer cells compared to those in the non-neoplastic ductal epithelium ($p < 0.001$).

According to the expression of P4HA1 or P4HA2,

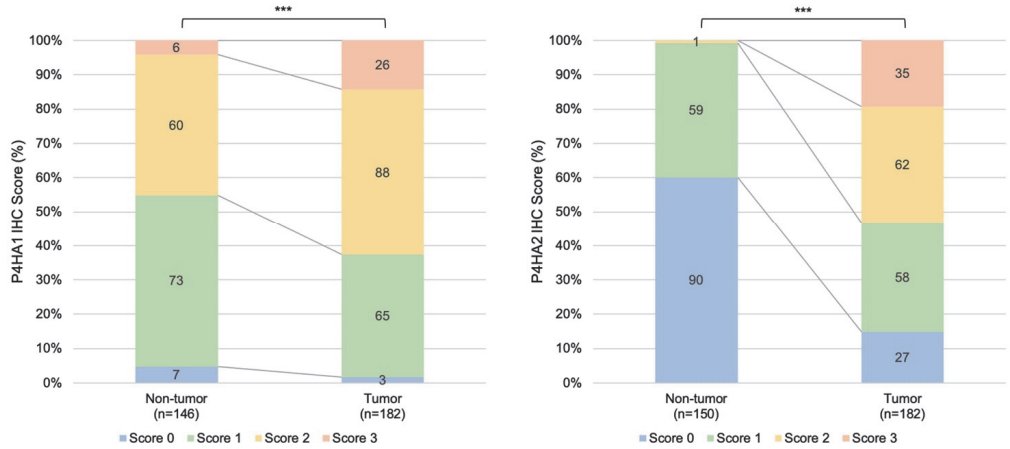

Fig. 2. Comparison of P4HA1 and P4HA2 scores in non-neoplastic ductal epithelium and tumor cells on tissue section for human breast cancer tissue samples. Both P4HA1 and P4HA2 were upregulated in tumor cells compared to that in the non-neoplastic ductal epithelium. *** $p < 0.001$

the patients were divided into high- and low-expression groups based on the median H-score, and the clinical and pathological data were compared (Table 1). For P4HA1, lower expression was statistically significantly correlated with negative lymphatic invasion ($p < 0.01$) and lymph node metastasis ($p < 0.05$). In contrast, the high-P4HA2 expression group showed a special histological type ($p < 0.05$), higher histological grade ($p < 0.001$), higher nuclear grade ($p < 0.001$), higher Ki67 labeling index ($p < 0.001$), p53 positivity ($p < 0.05$), and HER2/TN type ($p < 0.001$). These findings suggest that P4HA2 expression may be upregulated in high-grade tumors.

Correlation between expression of P4H, HIF, and EMT markers

Correlations were examined using the H-scores of the P4H, HIF, and EMT markers (Fig. 3). Positive correlations were found between P4HA1/P4HA2 and HIF-1 α/HIF-2 α ($r = 0.329$, $p < 0.0001$ and $r = 0.267$, $p < 0.0001$). Interestingly, P4HA2 expression showed a weak negative correlation with HIF-2 α expression ($r = -0.166$, $p < 0.05$). There was no evident correlation between P4HA1/HIF-1 α, P4HA1/HIF-2 α, and P4HA2/HIF-1 α ($r = 0.134$, $p = 0.06$ and $r = 0.064$, $p = 0.391$ and $r = 0.097$, $p = 0.185$). The EMT markers, Twist and ZEB1, showed no obvious correlation with the expression of either P4H or HIF markers. Both EMT markers were upregulated in metaplastic carcinoma (4/6) and invasive ductal carcinoma, solid type (2/6), indicating that they are expressed in histological types that cause EMT.

Outcome analysis

To evaluate the role of P4HA1 and P4HA2 expression in tumor cells, we divided patients into high- or low-expression groups (based on P4HA1 and P4HA2 expression), and the differences in prognosis were examined in terms of OS and RFS. There was no significant association between high-P4HA1 expression and low-P4HA1 expression groups in terms of OS (HR = 1.37, 95% CI: 0.47-3.95, $p = 0.562$, Fig. 4a). The same result was obtained for high-P4HA2 expression and low-P4HA2 expression groups (HR = 1.81, 95% CI: 0.62-5.28, $p = 0.27$, Fig. 4b). In terms of RFS, the time to recurrence tended to be shorter in the high-expression group compared to the low-expression

Table 1. Correlation between P4HA1 or P4HA2 expression in tumor cells with clinicopathological parameters in 182 cases.

Clinicopathological factors	Total number	P4HA1 expression in tumor epithelial cells		*P* value	P4HA2 expression in tumor epithelial cells		*P* value
		Low (n = 113) N. (%)	High (n = 69) N. (%)		Low (n = 122) N. (%)	High (n = 60) N. (%)	
Age							
< 55 years	57	41 (71.9)	16 (28.1)	0.072	36 (63.2)	21 (36.8)	0.498
≧55 years	125	72 (57.6)	53 (42.2)		86 (68.8)	39 (31.2)	
Histological subtype							
Invasive ductal carcinoma	155	98 (63.2)	57 (36.8)	0.427	105	50	***p* < 0.05**
- Tubule forming type	11	8 (72.7)	3 (27.3)		8 (72.7)	3 (27.3)	
- Solid type	73	46 (63.0)	27 (37.0)		47 (64.4)	26 (35.6)	
- Scirrhous type	68	44 (62.0)	27 (38.0)		50 (70.4)	21 (29.6)	
Invasive lobular carcinoma	9	6 (66.7)	3 (33.3)		8 (88.9)	1 (11.1)	
Mucinous carcinoma	4	1 (25.0)	3 (75.0)		3 (75.0)	1 (25.0)	
Invasive micropapillary carcinoma	4	2 (50.0)	2 (50.0)		1 (25.0)	3 (75.0)	
Metaplastic carcinoma	4	4 (100)	0 (0)		0 (0)	4 (100)	
Adenoid cystic carcinoma	2	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	
Apocrine carcinoma	4	1 (25.0)	3 (75.0)		9 (100)	0 (0)	
Histological grade							
Ⅰ	27	21 (77.8)	6 (22.2)	0.143	25 (92.6)	2 (7.4)	***p* < 0.001**
Ⅱ	56	31 (55.4)	25 (44.6)		46 (82.1)	10 (17.9)	
Ⅲ	99	61 (61.6)	38 (38.4)		51 (51.5)	48 (48.5)	
Nuclear grade							
1	46	33 (71.7)	13 (28.3)	0.218	42 (91.3)	4 (8.7)	***p* < 0.001**
2	32	17 (53.1)	15 (46.9)		26 (81.2)	6 (18.8)	
3	104	63 (60.6)	41 (39.4)		54 (51.9)	50 (48.1)	
Ki 67 labering index							
< 30%	71	46 (64.8)	25 (35.2)	0.639	58 (81.7)	13 (18.3)	***p* < 0.001**
≧30%	111	67 (60.4)	44 (39.6)		64 (57.7)	47 (42.3)	
p53 expression *							
Negative	104	65 (62.5)	39 (37.5)	1	77 (74.0)	27 (26.0)	***p* < 0.05**
Positive	78	48 (61.5)	30 (38.5)		45 (57.7)	33 (42.3)	
HIF-1α expression							
Low	101	63 (62.4)	38 (37.6)	1	65 (64.4)	36 (35.6)	0.43
High	81	50 (61.7)	31 (38.3)		57 (70.4)	24 (29.6)	
HIF-2α expression							
Low	127	82 (64.6)	45 (35.4)	0.321	81 (63.8)	46 (36.2)	0.173
High	55	31 (56.4)	24 (43.6)		41 (74.5)	14 (25.5)	
Twist expression							
Negative	67	42 (62.7)	25 (37.3)	1	46 (68.7)	21 (31.3)	0.747
Positive	115	71 (61.7)	44 (38.3)		76 (66.1)	39 (33.9)	
ZEB1 expression							
Negative	174	108 (62.1)	66 (37.9)	1	118 (67.8)	56 (32.2)	0.442
Positive	8	5 (62.5)	3 (37.5)		4 (50)	4 (50)	
pN							
0	123	85 (69.1)	38 (30.9)	***p* < 0.05**	82 (66.7)	41 (33.3)	0.332
1	35	18 (51.4)	17 (48.6)		26 (74.3)	9 (25.7)	
2	11	4 (36.4)	7 (63.6)		8 (72.7)	3 (27.3)	
3	13	6 (46.2)	7 (53.8)		6 (46.2)	7 (53.8)	
Lymphatic invasion							
Negative	99	70 (70.7)	29 (29.3)	***p* < 0.01**	69 (69.7)	30 (30.3)	0.432
Positive	83	43 (51.8)	40 (48.2)		53 (63.9)	30 (36.1)	
Venous invasion							
Negative	139	90 (64.7)	49 (35.3)	0.21	96 (69.1)	43 (30.9)	0.354
Positive	43	23 (53.5)	20 (46.5)		26 (60.5)	17 (39.5)	
Invasion size							
< 20 mm	98	65 (66.3)	33 (33.7)	0.223	70 (71.4)	28 (28.6)	0.206
≧20 mm	84	48 (57.1)	36 (42.9)		52 (61.9)	32 (38.1)	
Surrogated intrinsic subtypes							
Luminal A	51	37 (72.5)	14 (27.5)	0.181	45 (88.2)	6 (11.8)	***p* < 0.001**
HER2	48	27 (56.2)	21 (43.8)		29 (60.4)	19 (39.6)	
TN	83	49 (59.0)	34 (41.0)		48 (57.8)	35 (42.2)	
pStage							
Ⅰ	86	60 (69.8)	26 (30.2)	0.306	63 (73.3)	23 (26.7)	0.334
Ⅱ	69	41 (59.4)	28 (40.6)		44 (63.8)	25 (36.2)	
Ⅲ	26	12 (46.2)	14 (53.8)		15 (57.7)	11 (42.3)	
Ⅳ	1	0 (0)	1 (100)		0 (0)	1 (100)	
Comedo necrosis							
Absence	120	80 (66.7)	40 (33.3)	0.107	85 (70.8)	35 (29.2)	0.138
Presence	62	33 (53.2)	29 (46.8)		37 (59.7)	25 (40.3)	
Intraductal carcinoma component							
Absence	23	18 (78.3)	5 (21.7)	0.109	19 (82.6)	4 (17.4)	0.101
Presence	159	95 (59.7)	64 (40.3)		103 (64.8)	56 (35.2)	

* Case with nuclear staining of more than 10 % of the tumor cells were considered positive.
Each marker was grouped into high and low expression groups based on median values, with P4HA1 at 150 and P4HA2 at 130 of their H-scores for the respective immunostaining.
P4HA1: prolyl-4-hydroxylase alpha subunit 1, P4HA2: prolyl-4-hydroxylase alpha subunit 2, HIF-1 α: hypoxia inducible factor 1 alpha, HIF-2 α: hypoxia inducible factor 2 alpha, HER2: human epidermal growth factor receptor 2, ZEB1: Zinc finger E-box binding homeobox 1, TN: triple negative

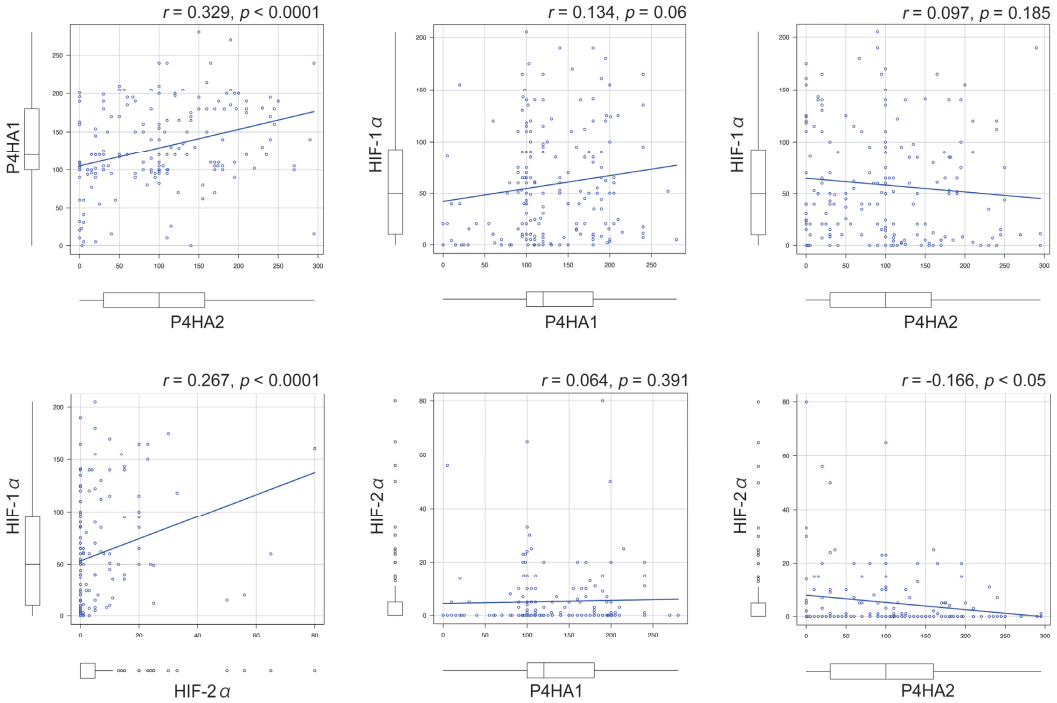

Fig. 3. Spearman correlation analysis of between the expression of P4H and HIF markers. Positive correlation was found between P4HA1-P4HA2 expression and HIF-1 α-HIF-2 α expression. Moreover, P4HA2 expression was negatively related to expression of HIF-2 α. Although no clear correlation was observed, there appeared to be a positive correlation trend between P4HA1 and HIF, and a negative correlation trend between P4HA2 and HIF-1 α.

group for both P4HA1 and P4HA2 (HR = 2.05, 95% CI: 0.87-4.84, $p = 0.09$; Fig. 4c and HR = 2.18, 95% CI: 0.92-5.19, $p = 0.07$, Fig. 4d).

In addition, we investigated OS and RFS by dividing the patients into four groups according to the combination of P4HA1 and P4HA2 expression: P4HA1 low/P4HA2 low, P4HA1 low/P4HA2 high, P4HA1 high/P4HA2 low, and P4HA1 high/P4HA2 high. In terms of OS, there was a trend toward a worse prognosis in the P4HA1 low/P4HA2 high group compared to that in the P4HA1 low/P4HA2 low group (HR = 3.84, 95% CI: 0.96-15.4, $p = 0.06$, Fig. 4e); however, there was no significant difference between the P4HA1 low/P4HA2 low group and P4HA1 high/P4HA2 low group (HR = 2.33, 95% CI: 0.58-9.31, $p = 0.23$) or between the P4HA1 low/P4HA2 low group and P4HA1 high/P4HA2 high group (HR = 1.91, 95% CI: 0.344-10.62, $p = 0.46$). In contrast, for RFS, the time to recurrence was shorter in the P4HA1 high/P4HA2 high group than in the P4HA1 low/P4HA2 low group (HR = 3.9, 95% CI: 1.24-12.34, $p < 0.05$, Fig. 4f). There was no significant difference between the P4HA1 low/P4HA2 low group and P4HA1 low/P4HA2 high groups (HR = 2.5, 95% CI: 0.71-8.88, p = 0.155) or between the P4HA1 low/P4HA2 low group and P4HA1 high/P4HA2 low group (HR = 1.99, 95% CI: 0.61-6.51, $p = 0.26$).

Univariate Cox regression analysis showed that high nuclear grade, lymph node metastasis, and the presence of comedo necrosis within intraductal components were associated with shorter RFS (Table 2). Multivariate Cox regression analysis showed that only comedo necrosis was associated with shorter RFS, and invasion size was shown to be an independent and significant factor affecting both OS

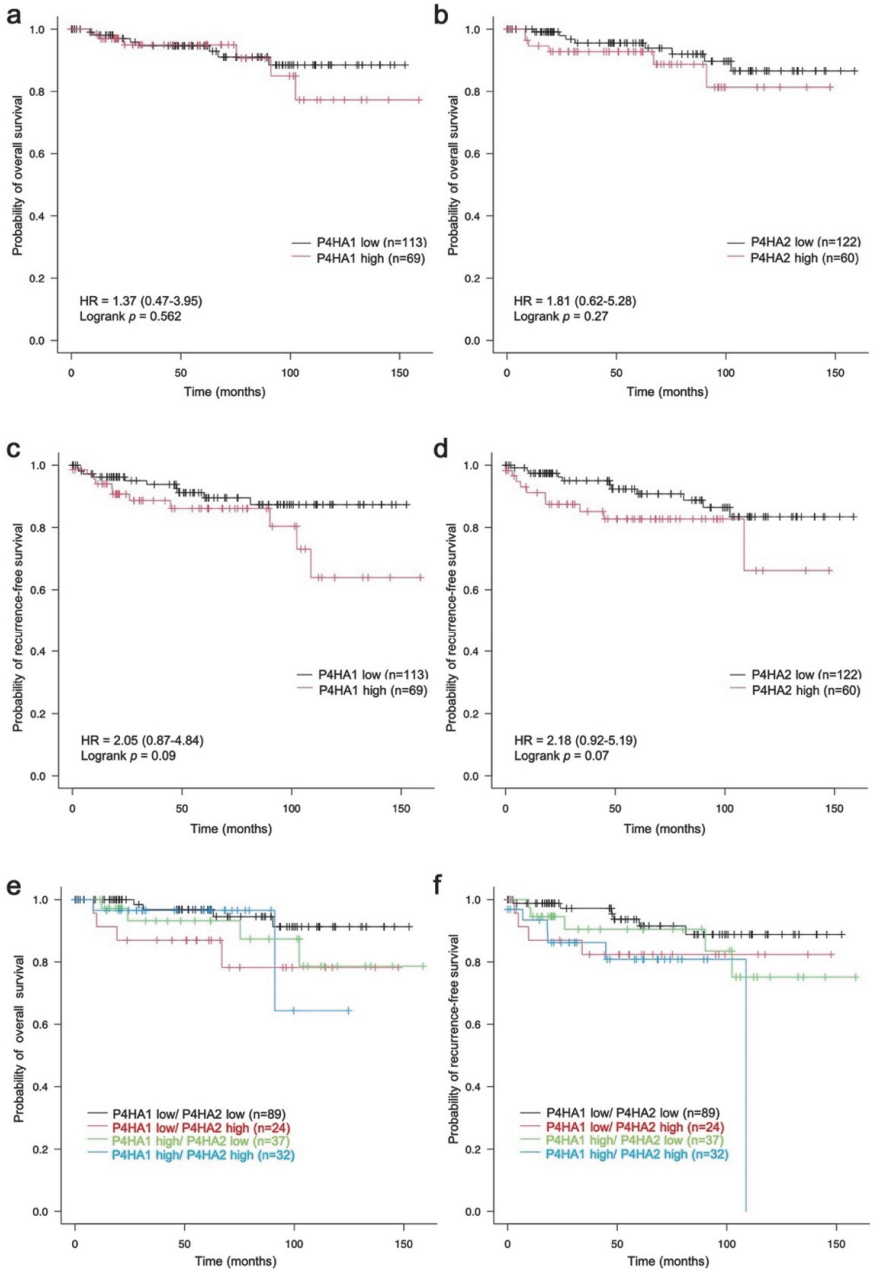

Fig. 4. Overall survival (OS) and recurrence-free survival (RFS) are shown. There was no significant difference between the high- and low-expression groups for either P4HA1 or P4HA2 in OS (a, b). In terms of RFS, the time to recurrence tended to be shorter in the high-expression group than in the low-expression group for both P4HA1 and P4HA2 (c, d). In a comparison of the four groups divided based on the expression pattern of P4H, there was a trend toward a worse prognosis in the P4HA1 low/P4HA2 high group compared with that in the P4HA1 low/P4HA2 low group in OS (HR = 3.84, 95% CI: 0.96-15.4, p = 0.06) (e), and the time to recurrence was shorter in the P4HA1 high/P4HA2 high group than in the P4HA1 low/P4HA2 low group (HR = 3.9, 95% CI: 1.24-12.34, $p < 0.05$) (f).

Table.2 Univariate cox regression analysis

	OS			RFS		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Age (<55 vs 55≦)	1.02	0.98 - 1.06	0.316	1.02	0.41 - 2.53	0.969
P4HA1 (high vs low)	1.37	0.47 - 3.95	0.562	2.05	0.87 - 4.84	0.091
P4HA2 (high vs low)	1.81	0.62 - 5.28	0.274	2.18	0.92 - 5.19	0.073
HIF-1 α (high vs low)	0.44	0.12 - 1.6	0.214	0.55	0.2 - 1.52	0.248
HIF-2 α (high vs low)	1.02	1.00 - 1.05	0.113	0.64	0.26 - 1.6	0.337
Twist (positive vs negative)	0.54	0.19 - 1.57	0.259	0.47	0.2 - 1.12	0.09
ZEB1 (positive vs negative)	2.29	0.3 - 17.6	0.425	1.37	0.18 - 10.3	0.757
Ki67 (<30% vs 30% ≦)	2.28	0.5 - 10.4	0.286	2.67	0.78 - 9.18	0.118
p53* (positive vs negative)	0.43	0.14 - 1.38	0.156	1.07	0.45 - 2.52	0.883
Histological grade (Ⅰ / Ⅱ vs Ⅲ)	1.35	0.42 - 4.38	0.616	2.02	0.73 - 5.55	0.174
Nuclear grade (1/2 vs 3)	3.02	0.67 - 13.6	0.15	3.45	1.01 - 11.8	**0.048**
Invasion size (<20mm vs 20mm ≦)	2.35	0.78 - 7.04	0.126	2.06	0.85 - 4.98	0.108
pN (0 vs 1-3)	2.22	0.79 - 6.43	0.14	2.48	1.05 - 5.9	**0.039**
Ly (0 vs 1)	1.49	0.52 - 4.29	0.462	1.53	0.64 - 3.63	0.336
V (0 vs 1)	1.2	0.38 - 3.19	0.723	1.56	0.63 - 0.88	0.335
Comedo necrosis (absence vs presence)	2.48	0.86 - 7.15	0.093	4.08	1.64 - 10.1	**0.002**
Subtype (non-TN vs TN)	2.85	0.78 - 10.4	0.113	1.78	0.71 - 4.46	0.221

Significant p values are shown in bold.
* Case with nuclear staining of more than 10 % of the tumor cells were considered positive.
OS: overall survival, RFS: recurrence-free survival, HR: hazard ratio, CI: confidence interval, P4HA1: prolyl-4-hydroxylase alpha subunit 1, P4HA2: prolyl-4-hydroxylase alpha subunit 2, HIF-1 α: hypoxia inducible factor 1 alpha, HIF-2 α: hypoxia inducible factor 2 alpha, ZEB1: Zinc finger E-box binding homeobox 1, Ly: lymphatic invasion, V: venous invasion, TN: triple negative

Table.3 Multivariate cox regression analysis

	OS			RFS		
	HR	95%CI	*p* value	HR	95%CI	*p* value
P4HA1	1.00	0.99 - 1.01	0.993	1.01	1.00 - 1.01	0.323
P4HA2	1.00	0.99 - 1.00	0.949	1.00	1.00 - 1.01	0.491
Ki-67	1.00	0.97 - 1.03	0.995	0.99	0.97 - 1.02	0.474
Nuclear grade	1.36	0.42 - 4.34	0.607	2.29	0.76 - 6.88	0.141
Comedo necrosis	2.49	0.81 - 7.74	0.113	3.09	1.21 - 7.93	**0.019**
Invasion size	1.07	1.04 - 1.1	**<0.001**	1.05	1.02 - 1.07	**<0.001**
pN	0.93	0.47 - 1.85	0.839	1.34	0.84 - 2.13	0.219

Significant p values are shown in bold.
OS: overall survival, RFS: recurrence-free survival, HR: hazard ratio, CI: confidence interval, P4HA1: prolyl-4-hydroxylase alpha subunit 1, P4HA2: prolyl-4-hydroxylase alpha subunit 2

and RFS (Table 3).

Expression levels of P4H and HIF proteins in human breast cancer tissues

From a total of 182 human breast cancer tissue samples, three patient samples representing each intrinsic subtype were chosen. The expression of P4H and HIF in human breast cancer tissues showed that HIF-1 α and HIF-2 α expression was remarkably lower than P4H expression, and there was a noticeable difference in expression between cases, even among the same subtypes (Fig. 5a). The expression of P4HA1 protein was similar in all subtypes, with no significant differences between intrinsic subtypes. However, P4HA2 protein expression was slightly higher in TN than in HER2 type. The expression levels of HIF-1 α, and HIF-2 α proteins were not significantly different between the subtypes (Fig. 5b).

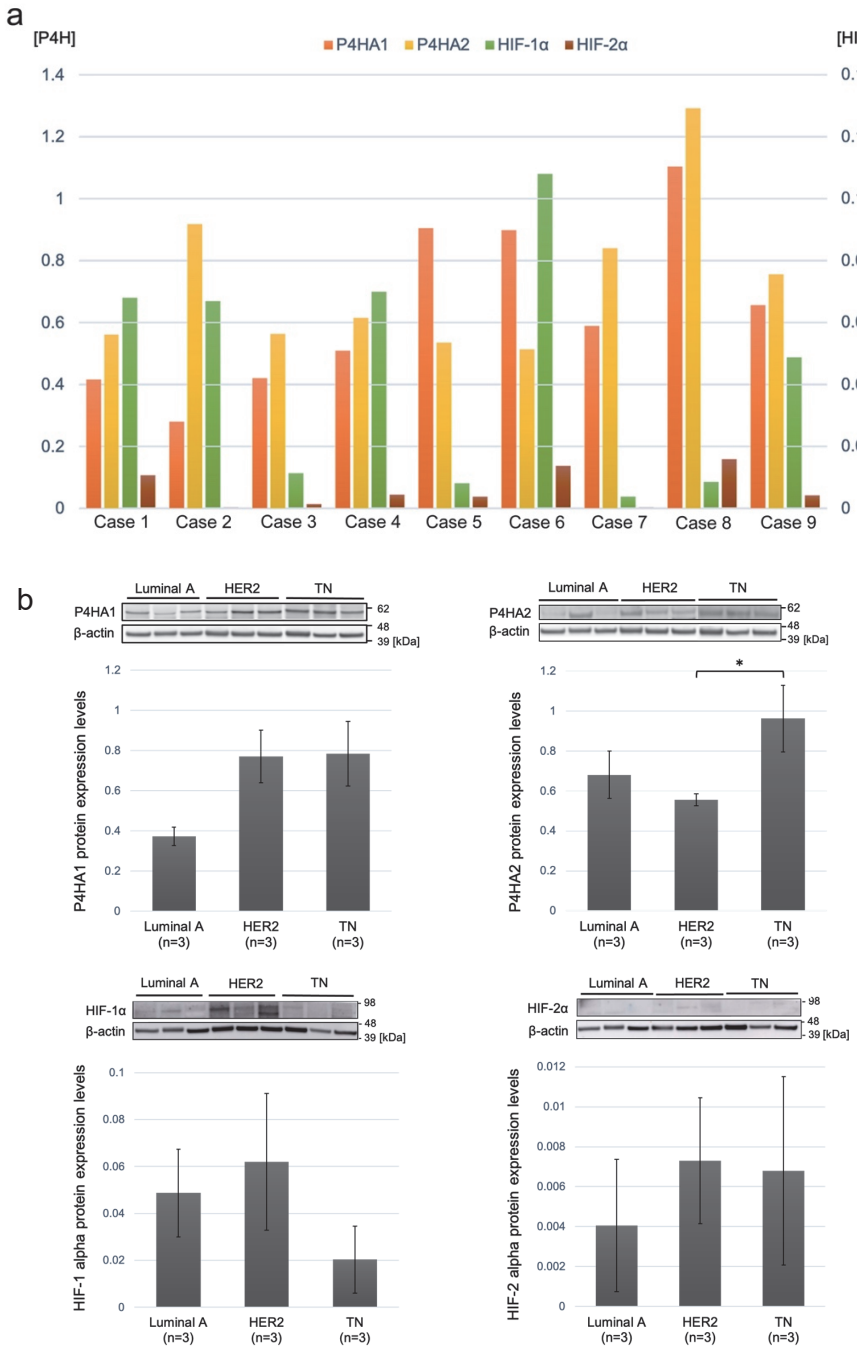

Fig. 5. Comparison of P4H and HIF protein expression levels in human breast cancer tissues. Cases 1-3 were of the luminal A type, cases 4-6 were of the HER2 type, and cases 7-9 were of the TN type, all of which showed differences in protein expression between cases (a). When compared among subtypes, P4HA2 protein expression was significantly higher in TN type than in HER2 type, but there were no significant differences in the expression levels of other markers between subtypes (b). Results are shown as mean ± SE. * $p < 0.05$

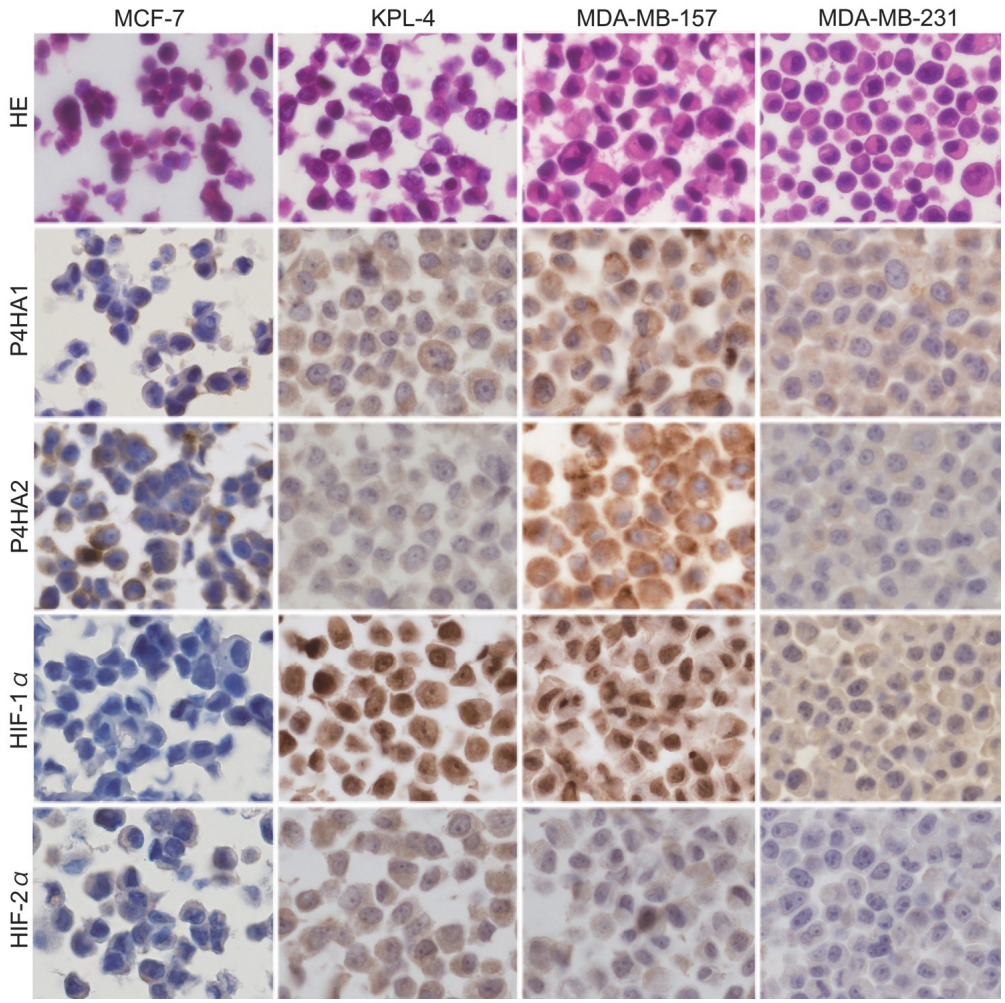

Fig. 6. Expression of P4HA1, P4HA2, HIF-1 α, and HIF-2 α through immunocytochemistry in breast cancer cell lines (MCF-7, KPL-4, MDA-MB-157, and MDA-MB-231) under normoxia. P4HA1 staining intensity was similar in all cell lines, while P4HA2 staining was strongly positive in MDA-MB-157. The nuclear expression of HIF-1 α and HIF-2 α was observed in KPL-4 and MDA-MB-157 under normoxia, but not in MCF-7 and MDA-MB-231 compared to the former.

P4H and HIF expression in breast cancer cell lines

Immunostaining of breast cancer tissue specimens showed a positive or negative correlation between P4H and HIF, which was comprehensively examined using cultured cells to investigate its significance.

Immunocytochemistry and P4HA1 staining intensity were similar in all cell lines under normoxia, whereas P4HA2 staining was strongly positive in TN cell lines, especially in MDA-MB-157 (Fig. 6). Nuclear expression of HIF-1 α and HIF-2 α was observed in KPL-4 and MDA-MB-157 cells under normoxia but not in MCF-7 and MDA-MB-231 cells. On the other hand, breast cancer cell lines cultured under hypoxia showed that the staining of P4HA1 was similar in both cell lines, but the staining intensity was slightly stronger than that under normoxia, suggesting that P4HA2

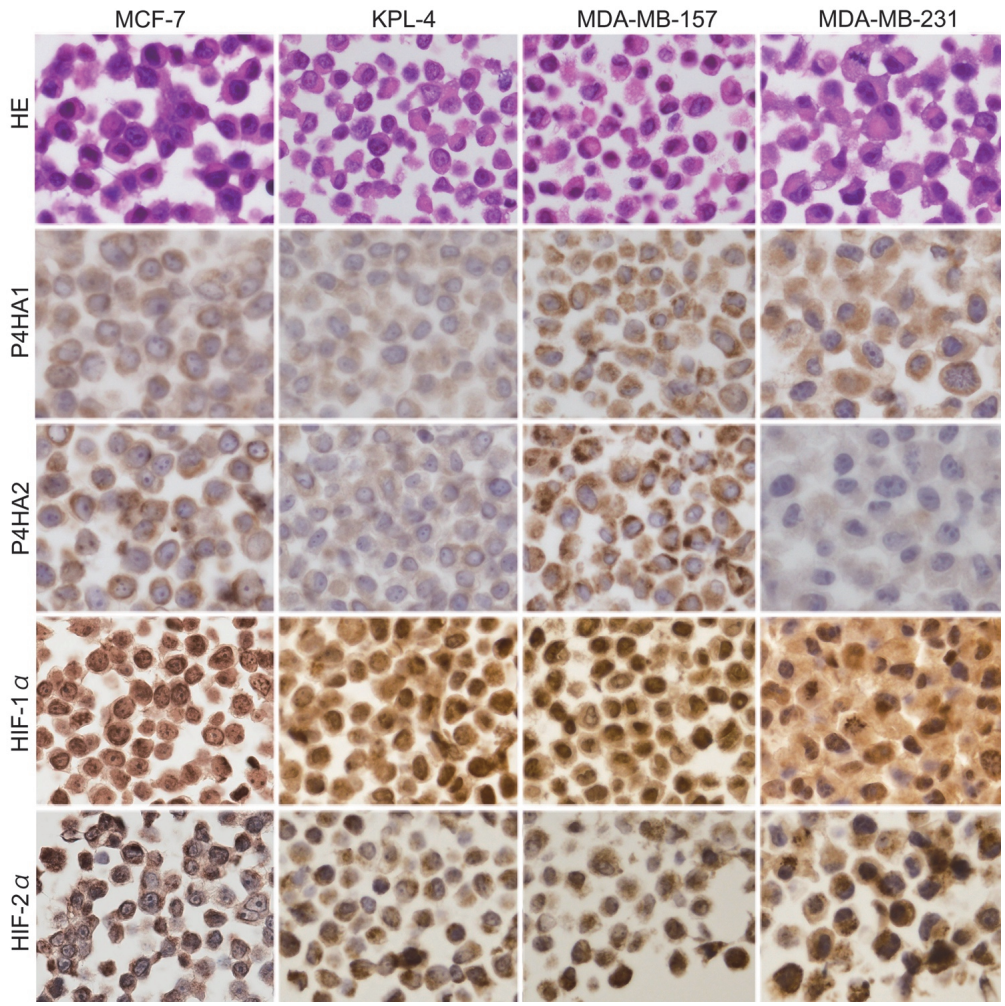

Fig. 7. Expression of P4HA1, P4HA2, HIF-1 α, and HIF-2 α through immunocytochemistry in breast cancer cell lines under hypoxia. Both P4H and HIF expression levels are enhanced in hypoxia compared with that in normoxia, although the degree of enhancement varies depending on the cell type.

is strongly positive in MCF-7 and MDA-MB-157 cells. HIF-1 α showed strong nuclear positivity in all cell lines, whereas HIF-2 α showed increased nuclear or cytoplasmic positivity in all cell lines compared to that under normoxia (Fig. 7). These results indicate that both P4H and HIF expression levels are enhanced under hypoxia compared to those under normoxia, although the degree of enhancement varies depending on the cell type.

In addition, we compared the changes in protein expression levels of P4HA1, P4HA2, HIF-1 α, and HIF-2 α in four different breast cancer cell lines using protein solutions extracted from cell lines cultured under normoxia and hypoxia for 4, 24, 48, and 72 h. Under normoxia, P4HA2 protein expression was significantly higher in MDA-MB-157 cells than in MCF-7 cells ($p < 0.05$). There were no significant differences in the expression levels of other proteins among the breast cancer cell lines (Fig. 8a). Next, we examined whether the degree of increase in the expression of various proteins varied among the cell lines over time in

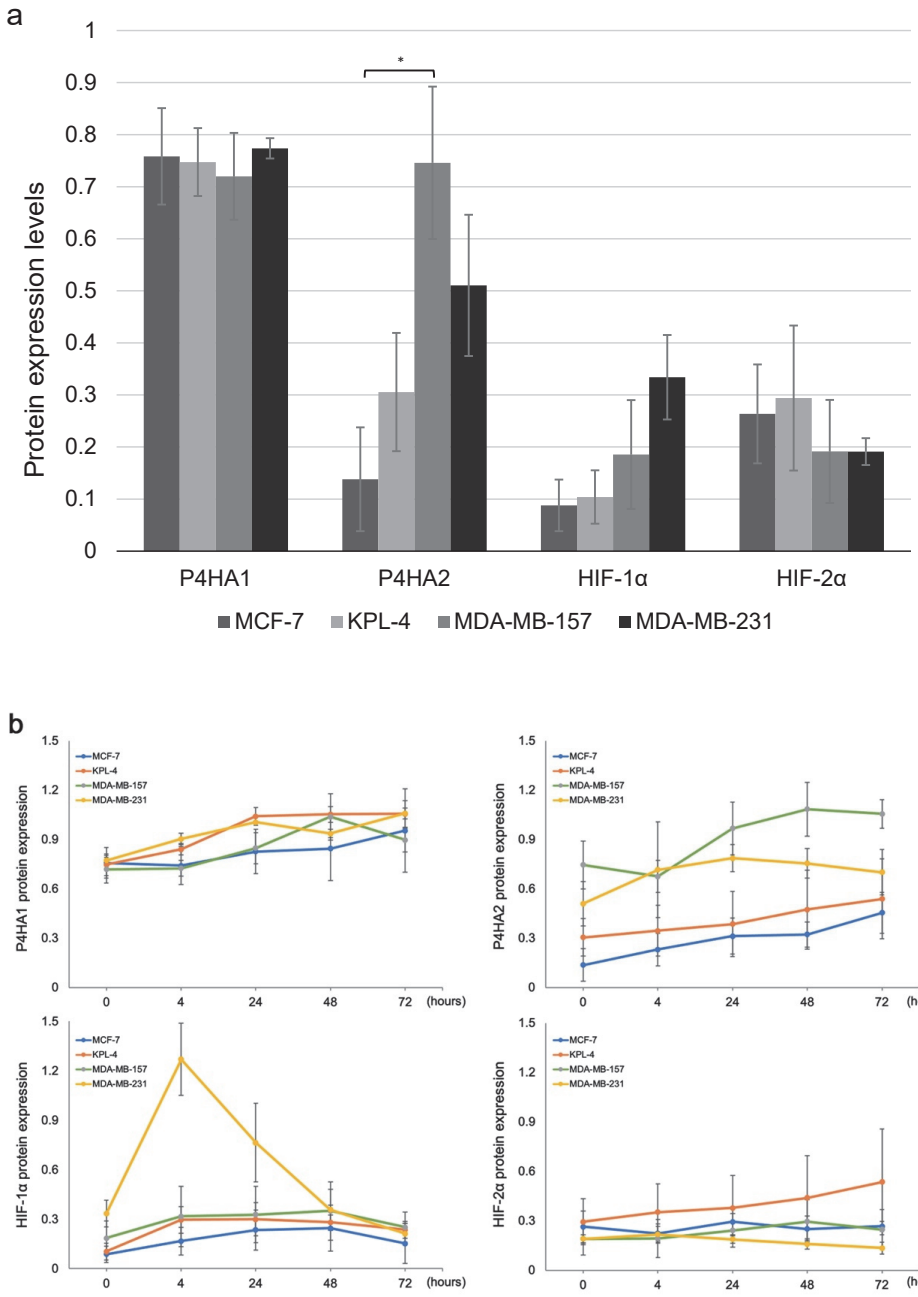

Fig. 8. Comparison of P4H and HIF protein expression levels in each breast cancer cell line. Under normoxia, P4HA2 protein expression was significantly higher in MDA-MB-157 than in MCF-7 cells, but there were no significant differences in P4HA1, HIF-1 α, and HIF-2 α levels between other cell lines (a). Comparison of protein expression levels at different time points showed that the protein expression of P4H tended to increase with prolonged hypoxia in all cell lines, and HIF protein expression showed a tendency to peak within 4-48 h, although some cell lines showed a steady increase in expression compared to P4H protein expression (b). Results are shown as mean ± SE. * $p < 0.05$.

hypoxic cultures (Fig. 8b). The results showed that P4HA1, P4HA2, and HIF-1α expression levels increased with increasing exposure time to hypoxia (all $p < 0.0001$), and the degree of increase in protein expression was significantly greater in MDA-MB-231 cells than in the other cell lines. There was no significant difference in the amount of increase in HIF-2α levels even after continuous hypoxia ($p=0.296$), and there was no significant difference in the amount of increase in HIF-2α levels among cell lines ($p=0.278$).

DISCUSSION

P4HA1 and P4HA2 were upregulated in tumor cells compared with those in non-tumor cells. However, P4HA1 was more strongly expressed in non-tumor cells than P4HA2. This may be because P4HA1 is an enzyme present in most cells in the human body[13, 14]. In contrast, P4HA2 is a cell-specific enzyme present in bone and cartilage cells; therefore, it is found at low levels in normal mammary tissue and is highly expressed only when cancer develops. This suggests that the expression of P4HA2 may be more tumor-specific than that of P4HA1. In terms of RFS, high P4HA1 and P4HA2 expression was associated with a higher risk of recurrence, which is consistent with the results of a previous study showing that P4HA2 induces cancer progression and metastasis[26]. A comparison between the four groups combining P4HA1 and P4HA2 expression showed that P4HA2 expression may contribute more to OS than P4HA1 expression, although the difference was not statistically significant. In terms of RFS, the high expression of both P4HA1 and P4HA2 was associated with a higher risk of recurrence. However, these results may be limited because it was not possible to investigate the prognosis of all patients.

The relationship between the expression of P4H, a major enzyme in collagen biosynthesis, and the expression of EMT transcription inducers was unclear in this study. However, this study only compared Twist and ZEB-1, with scope to examine other markers as well. At the cultured cell level, it has been reported that collagen, especially type I collagen, is important as a collagen that induces EMT[12]. In this study, we did not examine the type of collagen in the tumor stroma, but it may be related to type II collagen, especially since P4HA2 is an enzyme present in osteocytes and chondrocytes and it may not be directly related to EMT induction. A new role for P4H may be indicated by examining the characteristics of collagen deposited in the tumor stroma.

Regarding P4H and HIF, immunohistochemical staining of breast cancer cases showed that P4HA2 expression showed a weak negative correlation with HIF-2α expression (Fig. 3). Although no clear correlation was observed, there appeared to be a positive correlation trend between P4HA1 and HIF, and a negative correlation trend between P4HA2 and HIF-1α. To investigate this significance in detail, we performed experiments using four breast cancer cell lines. In cultured cells, both P4H and HIF were positively correlated with hypoxia. The discrepancy between the tissue and cultured cell results may be due to a shift in the peak expression of P4H and HIF. HIF expression was not significantly upregulated under normoxia, but it was decreased in most cell lines after 24-48 h (Fig. 8). Under normoxia, HIF-α is degraded via the ubiquitin-proteasome system by oxygen-dependent dioxygenases (PHDs), which act in a manner dependent on the presence of α-KG and oxygen. However, under hypoxia, PHD metabolism is suppressed and HIF-α protein is not degraded and accumulates intracellularly. This induces downstream gene expression, which is known to induce vascular endothelial growth factor and inducible nitric oxide synthase [27]. P4H is known to compete with PHD, a key enzyme in HIF-α metabolism, and has a 3-fold higher affinity for α-KG and 6-fold higher affinity for oxygen than

PHD[28]. Therefore, it is expected that under hypoxia, the HIF pathway will first be cut off, HIF proteins will accumulate, and then the P4H pathway will become less functional. Temporal differences in the peak expression of HIF and P4H may have occurred in breast cancer cells cultured under hypoxia. In addition, the positive correlation between P4HA1 and HIF and the opposite negative correlation between P4HA2 and HIF in breast cancer cases suggest that P4HA2 may have an inhibitory effect on HIF. As HIF-1 α and HIF-2 α have a gas pedal and brake relationship[27], we expect that P4HA1 and P4HA2 may also have such a relationship, which needs to be elucidated by detailed studies in the future.

CONCLUSION

The present study showed that P4H expression is upregulated when ductal epithelial cells become cancerous and that increased P4HA2 expression is a useful indicator of malignant transformation and may be a predictor of recurrence risk. The differences in the correlation between P4H and HIF expression in breast cancer tissues and cultured cells may be due to differences in the metabolic or regulatory mechanisms of P4H and HIF. Future studies on collagen in the tumor stroma are needed to elucidate the role of P4H.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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