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Profiling and Comparative Analysis of Glycoproteins in Hs578BST and Hs578T and Investigation of Prolyl 4-Hydroxylase Alpha Polypeptide II Expression and Influence in Breast Cancer Cells

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Abstract—To identify potential cancer related glycoproteins in breast cancer cells, we enriched N-linked glycoproteins by lentil lectin from the human breast cancer cell line Hs578T and the normal breast cell line Hs578BST for proteomic comparison. Glycoproteins were separated and compared by two-dimensional electrophoresis. Twenty-four glycoproteins were identified that expressed remarkably differently, among which nine were involved in the progress of collagen synthesis. Prolyl 4-hydroxylase alpha polypeptide II (P4HA2) expression and influence in breast cancer was further investigated. Immunohistochemistry revealed that P4HA2 was upregulated in breast tumor cells compared with its adjacent normal tissues. Moreover, overexpression and RNA interference of P4HA2 showed that P4HA2 expression suppressed cell proliferation and migration in Hs578T *in vitro*.

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Key words: glycoproteomics, lectin, breast cancer, P4HA2

Glycosylation is one of the most prevalent and abundant posttranslational modifications on eukaryotic proteins [1, 2]. This modification is shown to play crucial roles in various biological processes [3]. Aberrant glycosylation has been observed in essentially all types of experimental and human cancers and has been extensively investigated to determine its relationship with oncogenic transformation, proliferation, and metastasis [4-7]. Monitoring glycoprotein alterations during the progression of cancer could help to evaluate tumor progression and further define the mechanisms involved in cancer. In recent years, proteomic technologies have been widely used to discover cancer-related proteins and identify candidate tumor markers [8, 9].

Lectins are a group of glycoproteins that have high specific affinity to carbohydrates. Lectins are considered to be ideal for probing carbohydrate structure and are applied in various methods of detection and purification of glycoproteins. Lentil lectin (LCA) predominantly recognizes glycoproteins and glycopeptides containing structures including Fuc α1-6 GlcNAc, α-D-Glc, and α-D-Man [10, 11]. In a human liver glycoproteomic study, LCA was used for special capture and identification of α1-6-fucosylated proteins [12].

In breast tumors, abnormal glycosylation has been demonstrated as a remarkable alteration [13]. In this study, Sepharose 4B-LCA was employed for the isolation of glycoproteins from Hs578T breast cancer cells and Hs578BST normal breast cells. The profiling of proteins was analyzed using two-dimensional electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS). A group of glycoproteins that were significantly differentially expressed in the two cell lines was identified, and nine of them were correlated to collagen formation including protein synthesis and posttranslational modifications. These included collagen prolyl 4-hydroxylases (P4Hs), which participate in the catalysis and formation of 4-hydroxyproline residues and have a central role in stabilizing the collagen triple helix.

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In vertebrates, collagen P4Hs consists of $2\alpha2\beta$ tetramers and forms three isoenzymes depending on the type of catalytic α subunit, which contains the peptide substrate binding domain and the enzymatic active site. The β subunit of P4Hs maintains the α subunit in a soluble and active form [14]. In the three isoforms of the alpha P4H chain, P4HA1 is the most prevalent form expressed in most of cell types, and P4HA2 is found predominantly in chondrocytes, osteoblasts, and capillary endothelial cells, while P4HA3 is found at a much lower level [15]. Recently, P4HA2 expression has been reported to be altered in diverse tumor types including in oral cavity squamous cell carcinoma and papillary thyroid cancer, and it is thought to be involved in cell behaviors [16, 17]. In this study, we focused on P4HA2 and investigated its influence on the proliferation and migration of the breast cancer cell line Hs578T.

MATERIALS AND METHODS

Sample preparation. HS578T and HS578BST were purchased from the ATCC (also termed HTB-126 and HTB-125, respectively). Collected cells were lysed by sonication, and the homogenate was incubated on ice for 1 h and then centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was collected and applied to a Sepharose 4B-LCA column (GE Healthcare, USA), pre-equilibrated with binding buffer (20 mM Tris, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$). For each sample, 5 ml lysis supernatant (about 20 mg of protein, as quantified by BCA assay) was dialyzed against binding buffer overnight at 4°C and loaded onto the column. After incubation overnight at 4°C, the column was washed three times with binding buffer. The glycoproteins were then collected in 5 ml of elution buffer (20 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100, 1% deoxycholate, 0.1 M α-D-methylmannoside and 0.1 M α-D-methylglucoside).

2-DE separation. A total of 200 μg of glycoproteins was precipitated using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, USA) and dissolved in 400 μl of rehydration solution (8 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer and 18 mM DTT) for each gel. The proteins were loaded onto pH 4-7 and pH 6-9 IPG strips (17 cm; GE Healthcare). Isoelectric focusing, equilibration, and SDS-PAGE were carried out according to the manufacturer's instructions. Then the separated proteins were visualized by silver staining using a Fast Silver Stain Kit (Beyotime, China).

Protein digestions and MS identification. Protein spots were excised from the preparative gels, destained, and in-gel digested with sequencing-grade trypsin (Promega, USA). The peptides were extracted with 0.1% TFA in 60% ACN and lyophilized. MS and MS/MS spectra were obtained using the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, USA) operating in a result-dependent acquisition mode. The MS together with the MS/MS spectra were searched against the UniProtKB/SwissProt database using the software GPS Explorer version 3.6 and MASCOT version 2.1.

Real-time PCR and Western blot analysis. Real-time PCR was carried out using the SYBR Premix EX *Taq* II kit (TaKaRa, Japan), and data were acquired and analyzed by an Eppendorf Mastercycler ep realplex system (Eppendorf, Germany). The primers used were P4HA1 sense: 5′-CAA AGA CTG GGG AAG CAG AA-3′, antisense: 5′-CCT CTC GTC CCA CTT TCC A-3′; P4HA2 sense: 5′-AGC TCA GGA CAC CAA ACC AG-3′, antisense: 5′-CTT CCT GCC TCA GGT ATT GC-3′; GAPDH sense: 5′-GTG AAG GTC GGA GTC AAC G-3′, antisense: 5′-TGA GGT CAA TGA AGG GGT C-3′.

Whole cell lysate was prepared using RIPA lysis buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). After electrophoretic separation by 8% SDS-PAGE and transfer to a PVDF membrane, the proteins were detected with antibodies (P4HA1 antibody was obtained from Sigma (USA), P4HA2 antibody from Abgent, P4HB and second antibodies from Santa Cruz (USA)).

Immunohistochemistry. Immunohistochemistry was used to investigate the expression of P4HA2 in breast cancer. Paraffin block tissue chips (Shanghai Outdo Biotech Co., Ltd., China) were detected with P4HA2 antibody (Abgent, USA) diluted 1 : 100. The intensity of immunohistochemical immunoreactivity was graded as follows: 1, weak reactivity; 2, moderate reactivity; and 3, strong reactivity. The proportion of immunoreactive cells was categorized as: 0: <5% of cells reactive, 1: 5-25% of cells reactive, 2: 26-50% of cells reactive, 3: 51-75% of cells reactive, 4: >75% of cells reactive. The total score for each case was calculated by adding the proportion and intensity scores. Student's *t*-test was used to analyze the difference of P4HA2 expression and a value of $P < 0.05$ was considered statistically significant.

Overexpression and RNA interference of P4HA2. The *P4HA2* gene was cloned from HS578T cDNA. Primers employed were P4HA2 sense: 5′-CGC GGA TCC ATG AAA CTC TGG GTG T-3′, antisense: 5′-CCG CTC GAG TCA GTC AAC TTC TGT T-3′. After digesting with *Bam*HI and *Xho*I, the amplified fragments were ligated into the pcDNA3.1(+) vector and were designated pcDNA3.1(+)-P4HA2. Then the recombinant plasmid was transfected into Hs578T cells using polyethyleneimine (PEI) transfection reagent. The RNA interference was carried out using P4HA2 shRNA and control shRNA (Santa Cruz) following the manufacturer's instructions. The overexpression and interference of P4HA2 were determined by Western blot.

Cell proliferation and transwell migration assay. After transfection with pcDNA3.1(+) or shRNA plasmids for 72 h, the cells were collected and counted three times for

the cell proliferation study as presented below. The cells (10^5 cells transfected with control and recombined pcDNA3.1(+) or 4×10^5 cells transfected with shRNAs) were suspended in DMEM medium and placed in the upper chamber of the transwell unit, which was immersed into the lower chamber containing DMEM medium (0.5% FBS). After 6 h of incubation, the cells were dyed with crystal violet solution, and the upper side of the filter removed and gently wiped with a cotton swap. Cell migration was determined by counting the cells that migrated to the lower side of the polycarbonate filters.

RESULTS

Isolation of glycoproteins. Using Sepharose 4B-LCA affinity chromatography, we enriched the glycoproteins from HS578T breast cancer cells and HS578BST normal cells. As shown in Table 1, after two separations, 3.9 and 4.1% yield of total glycoproteins was isolated from the HS578BST cell line while 7.9 and 7.2% were isolated from HS578T, which suggests that the method was repeatable. In addition, the glycoproteins captured from HS578T cells were nearly 2-fold higher than those from HST578BST cells, indicating that the breast cancer cells underwent more extensive glycosylation compared with normal breast cells.

2-DE comparison. The glycoproteins isolated from HS578BST and HS578T were separated by 2-DE. To identify more protein spots with higher precision, we used different pH range IPGs (pH 4-7, 6-9) for the separation of acidic and basic proteins. Figure 1 (see color insert) shows the protein separation of the two samples. A total of 84 spots in the pH 4-7 gels and 13 spots in the pH 6-9 gels were found that varied more than 2-fold. In addition, 24 of these spots were eventually identified, among which seven were upregulated in Hs578BST cells and 17 in Hs578T cells (Table 2). A database search was conducted to examine the glycosylation of the identified proteins, and 21 proteins were found to be annotated as glycosylated in the UniProtKB database.

According to the cellular process, we classified these glycoproteins into different functional categories. Among them, seven glycoproteins are involved in cell adhesion and six glycoproteins function in oxidation–reduction processes. Furthermore, it was noticeable that a group of glycoproteins were involved in the synthesis of collagen, and most of them were upregulated in Hs578T cells. COL6A1 and COL6A2 belong to the type VI collagen family and function as a component of collagen VI [18]. P4HA1, P4HA2, and LEPRE1 (also known as P3H1) participate in proline hydroxylation. PLOD1 and PLOD2 participate in lysine hydroxylation of procollagen. Hydroxyproline and hydroxylysine are important post-translational modifications that play an important role in collagen chain forming a proper helical structure and stable intermolecular crosslinks [19, 20]. FKBP10 and FKBP10 belong to the peptidyl-prolyl *cis-trans*-isomerase (FKBP) family and were demonstrated to catalyze the refolding of collagen as peptidyl-prolyl isomerase (PPIase) [21, 22]. Taken together, these results indicated an abnormal synthesis of collagen in Hs578T cells.

Table 1. Glycoproteins captured from Hs578BST and Hs578T cells

Cell line	Loading, mg	Elution, mg	Glycoproteins, %
Hs578BST	17.6	0.715	4.1
	19	0.795	3.9
Hs578T	16	1.265	7.9
	17.5	1.245	7.2

Validation of P4HA2 expression in cells and further investigation in breast tumor tissues. From the glycoproteins identified, P4HA1 and P4HA2, which participate in proline hydroxylation, were found to be differentially expressed in Hs578BST and Hs578T cells (Fig. 2a). As analyzed with PDQuest (v8.0), P4HA1 in Hs578T cells was downregulated (>3-fold), while P4HA2 was upregulated (>6-fold) compared with Hs578BST. This result was further confirmed by real-time PCR and Western blotting. As shown in Fig. 2b, P4HA2 increased more than 10-fold in Hs578T cells by real-time PCR, but P4HA1 increased only 1.7-fold in Hs578BST ($P < 0.01$). Western blotting showed a similar result, a moderately weaker band for P4HA1 was observed in Hs578T cells and an almost nonexistent band for P4HA2 was observed in Hs578BST (Fig. 2c). The β subunit of prolyl 4-hydroxylase (P4HB) was not found to be obviously different between the cells. These results suggest the differential expression of the alpha subunit of P4H at both the transcriptional and translational levels. The expression of P4HA2 was at a much lower level in Hs578BST cells compared with Hs578T cells, while P4HA1 was only slightly different between the two.

P4HA2 expression in breast tumors and homologous normal breast tissues was investigated further using immunohistochemical analysis. As shown in Fig. 3 (see color insert), P4HA2 was generally expressed in tumor cases and mainly localized in the cytoplasm. Positive staining was observed throughout the cancer tissues and part of the normal tissues and in most cases. The statistical analysis indicated the expression of P4HA2 was significant higher in breast tumor tissues ($P < 0.01$).

Influence of P4HA2 on proliferation and migration ability of Hs578T cells. To further investigate the influence of P4HA2 on the proliferation and migration of breast cancer cells, overexpression and RNA interference experiments of targeted P4HA2 were carried out. After shRNA-P4HA2 and pcDNA3.1-P4HA2 plasmids were

Table 2. Identification of glycoproteins significantly altered in gels of Hs578BST and Hs578T cells

Spot	Gene ID	Protein	Mol. weight	Gly-sites	Score	Biological process
B1	IPI00294578	TGM2 Isoform 1 of Protein-glutamine gamma-glutamyltransferase 2	78420.1	2	121	positive regulation of cell adhesion
B2	IPI00218682	P4HA1 Isoform 2 of prolyl 4-hydroxylase subunit alpha-1	61214	2	80	oxidation–reduction process
B3	IPI00645452	TUBB Tubulin, beta	48135.1	2	503	microtubule-based movement
B4	IPI00334190	STOML2 Stomatin-like protein 2	38624.2	0	106	receptor binding
B5	IPI00879747	ERLIN1 ER lipid raft associated 1	31171.4	2	73	ER-associated protein catabolic process
B6	IPI00024145	VDAC2 Isoform 2 of voltage-dependent anion-selective channel protein 2	30849.2	2	111	ion transport
B7	IPI00220327	KRT1 Keratin, type II cytoskeletal 1	66170.1	2	226	regulation of angiogenesis
T1	IPI00013976	LAMB1 Laminin subunit β-1	205178.3	11	295	cell adhesion
T2	IPI00298281	LAMC1 Laminin subunit γ-1	183190.7	11	412	cell adhesion
T3	IPI00291136	COL6A1 Collagen α-1 (VI) chain	109602.4	5	909	cell adhesion
T4	IPI00307162	VCL Isoform 2 of vinculin	124292	5	59	cell adhesion
T5	IPI00215995	ITGA3 Isoform α-3A of integrin α-3	117735.5	11	52	cell adhesion
T6	IPI00304840	COL6A2 Collagen α-2 (VI) chain	109709.4	5	296	cell adhesion
T7	IPI00163381	LEPRE1 Isoform 1 of prolyl 3-hydroxylase 1	84196.4	3	320	oxidation–reduction process
T8	IPI00182373	P4HA2 Isoform IIa of prolyl 4-hydroxylase subunit α-2	60993.7	2	155	oxidation–reduction process
T9	IPI00337495	PLOD2 Isoform 2 of procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	87784.1	6	175	oxidation–reduction process
T10	IPI00011200	PHGDH D-3-phosphoglycerate dehydrogenase	57355.7	2	136	oxidation–reduction process
T11	IPI00943008	PLOD1 Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	84067.6	4	219	oxidation–reduction process
T12	IPI00182126	FKBP9 Peptidyl-prolyl *cis-trans*-isomerase FKBP9	63499.7	4	403	protein folding
T13	IPI00303300	FKBP10 Peptidyl-prolyl *cis-trans*-isomerase FKBP10	64717.4	5	640	protein folding
T14	IPI00020599	CALR Calreticulin	48282.9	0	102	regulation of apoptosis
T15	IPI00645452	TUBB Tubulin, β	48135.1	2	690	microtubule-based movement
T16	IPI00644620	TUBB3 35 kDa protein	34806.8	2	100	microtubule-based movement
T17	IPI00440493	ATP5A1 ATP synthase subunit α, mitochondrial	59827.6	0	131	ATP synthase

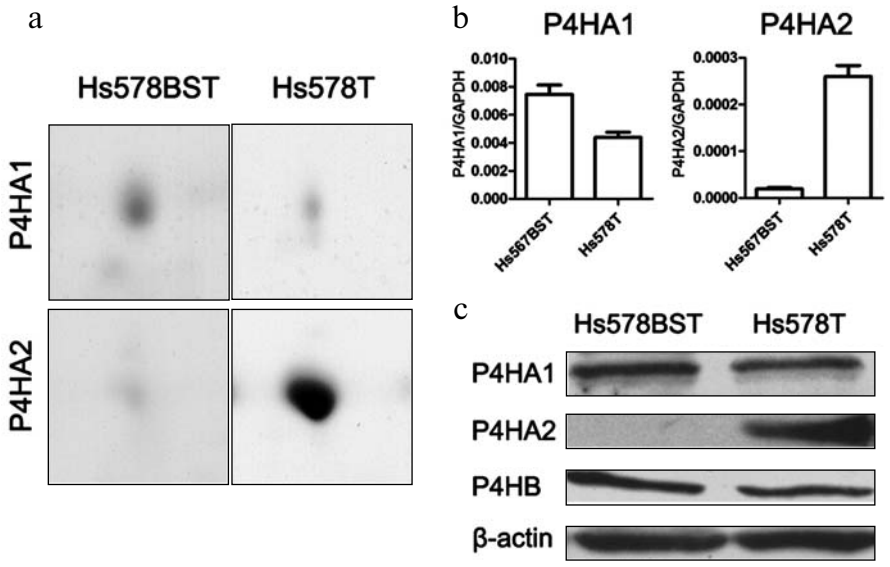

Fig. 2. Comparative analysis of P4HA1 and P4HA2 expression. a) The spots of P4HA1 and P4HA2 are shown in the enlarged images of the 2-DE gels. b) Expression of P4HA1 and P4HA2 were analyzed by real-time PCR, the mRNA level being normalized to GAPDH. c) Western blotting was applied to investigate the amount of P4HA1, P4HA2, and P4HB. β-Actin was employed as an endogenous reference protein.

transfected into Hs578T, P4HA2 expression levels were detected by Western blotting. As shown in Fig. 4, the proteins were obviously up- or downregulated 72 h after transfection. Then, the correlation between the P4HA2 level and the cell behaviors of Hs578T was further investigated. RNA interference of P4HA2 could effectively increase the proliferation (50.1%, $P < 0.01$) and migration (30.7%, $P < 0.05$) of Hs578T cells *in vitro*, and overexpression of P4HA2 obviously reduced the proliferation (62.4%, $P < 0.01$) and migration (53%, $P < 0.01$).

DISCUSSION

Breast cancer is one of the most common cancers throughout the world, thus better understanding of its molecular mechanisms should help diagnosis, prognosis, and therapy of this disease. In our study, a proteomic comparison was applied for identifying cancer related glycoproteins. After LCA capture, nearly 2-fold more proteins were isolated from Hs578T breast cancer cells compared with normal Hs578BST cells. According to previous reports, breast cancer-associated alterations in glycosylation include fucosylations, sialylations, and GlcNAc β1-6 branching in N-linked oligosaccharides [23, 24]. Fucosylation was found to be increased in breast cancer, both in the core and the branched segments of N-glycans [25, 26]. The incorporation of fucose into glycoproteins was related with the malignant phenotype and was also proven to contribute to many of the fundamental oncologic properties of breast cancer cells [27]. Considering the affinity properties of LCA, the increased number of glycoproteins captured from Hs578T cells is expected to be partly contributed to by alteration in glycosylation levels, especially fucosylation, aside from the observed differences in protein expression.

As a component of the extracellular matrix, collagen is involved in the constitution of the cell microenvironment and affects cell adhesion. Among the glycoproteins that were identified, nine are involved in the synthesis of collagen. In addition to P4HA1, the other eight proteins were all found to be upregulated in Hs578T cells. COL6A1 has been demonstrated to be upregulated during the progress of tumor development in human astrocytomas and is believed to be involved in tumor invasiveness [28]. PLOD2 was reported to be overexpressed in cervical tumors and was identified as a factor contributing to poor prognosis for hepatocellular carcinoma patients [29, 30]. Proteomic and microarray analysis revealed that P4HA2 was related with metastasis and recurrence of oral carcinoma [16, 31]. The differential expression of these glycoproteins indicated the formation of abnormal collagens in Hs578T cells, which might be a potential feature and enough to affect the behavior of tumor cells.

P4HA1 and P4HA2 have similar enzymatic properties, but the ratio of K_i/K_m for the affinity of the inhibitor and the substrate toward the synthesis of collagen were different [32]. Other research has indicated that they function in markedly distinct fashion at the molecular and biological level [33]. In our study, it was found that the expression levels of P4HA1 and P4HA2 were different in Hs578BST and Hs578T cells. It was remarkable that P4HA2 was essentially not expressed in normal Hs578T breast cells, and hence, we focused our research on this

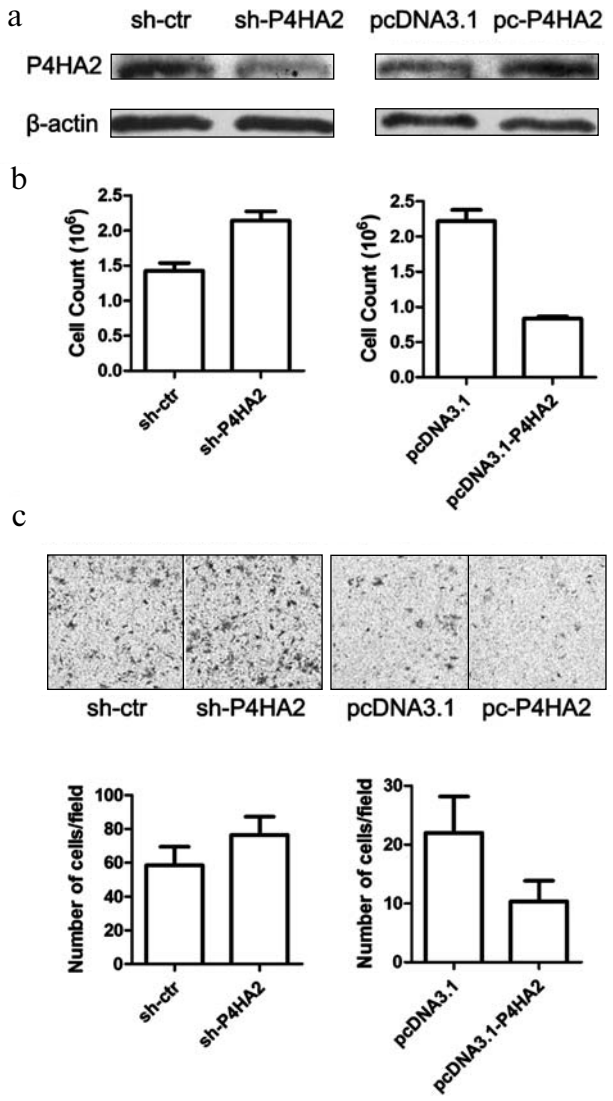

Fig. 4. Influence of P4HA2 on the behavior of Hs578T cells. Hs578T cells were transfected with shRNA-P4HA2 and pcDNA3.1-P4HA2 plasmids. Control shRNA and pcDNA3.1 plasmids were transfected as negative controls. a) Western blot detection of P4HA2 expression in transfected cells. b) Cells were counted 72 h after transfection ($P < 0.05$ in the shRNA matched group and $P < 0.01$ in the pcDNA3.1 matched group). c) The ability of cells to migrate was investigated by a transwell assay. Six random fields of view were selected, and the cell numbers are listed in the histogram ($P < 0.05$ for the shRNA matched group and $P < 0.01$ for the pcDNA3.1 matched group).

glycoprotein. Immunohistochemical analysis showed the prevalent expression of P4HA2 as 24 out of 30 tumor samples expressed P4HA2 with >75% population testing positive. In normal tissues, this number was reduced to 15 and P4HA2 expression was not even detected in four cases (data not shown). Therefore, it was considered that overexpression of P4HA2 was related to cancer progression in breast tumors; however it might not be an ideal marker for the diagnosis of breast cancer considering its high expression in parts of normal tissues.

We demonstrated that the level of P4HA2 negatively controlled the migration and proliferation of Hs578T cells *in vitro*. Tumor suppressor functions have also been proposed for different isozymes of P4H such as PHDs in many types of tumors [34]. On the other hand, the upregulation of PHD2 and P4HA2 has been shown to be associated with metastasis in head and neck squamous cell carcinoma [16, 35]. These results indicate that the activity of P4H plays an important role in tumor behavior; however, the effect was diverse depending on the isozyme of P4H involved and the cancer types. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that responds to hypoxia and promotes transcription of many genes associated with increased tumor growth, vascularization, and metastasis [36, 37]. Prolyl-4-hydroxylation is necessary for HIF-1 degradation [38]. This modification can be catalyzed by P4HA2, and the silencing of P4HA2 has been shown to promote HIF-1 activation [39]. Taken together, HIF-1 is believed to be a potential pathway by which P4HA2 can affect breast cancer cell behavior. Further study is necessary to confirm this hypothesis to gain a better understand of the mechanism of P4HA2 in breast cancer metastasis.

In conclusion, the application of lectin and proteomic techniques revealed a group of glycoproteins differentially expressed between Hs578T and Hs578BST cells. P4HA2 was demonstrated to be upregulated and found to inhibit cell proliferation and migration in breast cancer cells.

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REFERENCES

1. Bertozzi, C. R., and Kiessling, L. L. (2001) *Science*, **291**, 2357-2364.
2. Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A., and Dwek, R. A. (2001) *Science*, **291**, 2370-2376.
3. Varki, A. (1993) *Glycobiology*, **3**, 97-130.
4. Ng, R. C., Roberts, A. N., Wilson, R. G., Latner, A. L., and Turner, G. A. (1987) *Br. J. Cancer*, **55**, 249-254.
5. Kasbaoui, L., Harb, J., Bernard, S., and Meflah, K. (1989) *Cancer Res.*, **49**, 5317-5322.
6. Hakomori, S. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 10231-10233.
7. Ono, M., and Hakomori, S. (2004) *Glycoconj. J.*, **20**, 71-78.
8. Brewis, I. A., and Brennan, P. (2010) *Adv. Protein Chem. Struct. Biol.*, **80**, 1-44.
9. Steel, L. F., Mattu, T. S., Mehta, A., Hebestreit, H., Dwek, R., Evans, A. A., London, W. T., and Block, T. (2001) *Dis. Markers*, **17**, 179-189.
10. Kornfeld, K., Reitman, M. L., and Kornfeld, R. (1981) *J. Biol. Chem.*, **256**, 6633-6640.

11. Fang, X., and Zhang, W. W. (2008) *J. Proteomics*, **71**, 284-303.
12. Dai, Z., Fan, J., Liu, Y., Zhou, J., Bai, D., Tan, C., Guo, K., Zhang, Y., Zhao, Y., and Yang, P. (2007) *Electrophoresis*, **28**, 4382-4391.
13. Kim, E. H., and Misek, D. E. (2011) *Int. J. Proteomics*, **2011**, 601937.
14. Gorres, K. L., and Raines, R. T. (2010) *Crit. Rev. Biochem. Mol. Biol.*, **45**, 106-124.
15. Myllyharju, J., and Schipani, E. (2010) *Cell Tissue Res.*, **339**, 19-29.
16. Jarzab, B., Wiench, M., Fujarewicz, K., Simek, K., Jarzab, M., Oczko-Wojciechowska, M., Wloch, J., Czarniecka, A., Chmielik, E., Lange, D., Pawlaczek, A., Szpak, S., Gubala, E., and Swierniak, A. (2005) *Cancer Res.*, **65**, 1587-1597.
17. Chang, K. P., Yu, J. S., Chien, K. Y., Lee, C. W., Liang, Y., Liao, C. T., Yen, T. C., Lee, L. Y., Huang, L. L., Liu, S. C., Chang, Y. S., and Chi, L. M. (2011) *J. Proteome Res.*, **10**, 4935-4947.
18. Merlini, L., Angelin, A., Tiepolo, T., Braghetta, P., Sabatelli, P., Zamparelli, A., Ferlini, A., Maraldi, N. M., Bonaldo, P., and Bernardi, P. (2008) *Proc. Natl. Acad. Sci. USA*, **105**, 5225-5229.
19. Bienkowski, R. S., Baum, B. J., and Crystal, R. G. (1978) *Nature*, **276**, 413-416.
20. Vranka, J. A., Sakai, L. Y., and Bachinger, H. P. (2004) *J. Biol. Chem.*, **279**, 23615-23621.
21. Steinmann, B., Bruckner, P., and Superti-Furga, A. (1991) *J. Biol. Chem.*, **266**, 1299-1303.
22. Ishikawa, Y., Vranka, J., Wirz, J., Nagata, K., and Bachinger, H. P. (2008) *J. Biol. Chem.*, **283**, 31584-31590.
23. Goodarzi, M. T., and Turner, G. A. (1995) *Clin. Chim. Acta*, **236**, 161-171.
24. Dennis, J. W., and Laferte, S. (1989) *Cancer Res.*, **49**, 945-950.
25. Kyselova, Z., Mechref, Y., Kang, P., Goetz, J. A., Dobrolecki, L. E., Sledge, G. W., Schnaper, L., Hickey, R. J., Malkas, L. H., and Novotny, M. V. (2008) *Clin. Chem.*, **54**, 1166-1175.
26. Alley, W. R., Jr., Madera, M., Mechref, Y., and Novotny, M. V. (2010) *Anal. Chem.*, **82**, 5095-5106.
27. Listinsky, J. J., Siegal, G. P., and Listinsky, C. M. (2011) *Am. J. Transl. Res.*, **3**, 292-322.
28. Fujita, A., Sato, J. R., Festa, F., Gomes, L. R., Oba-Shinjo, S. M., Marie, S. K., Ferreira, C. E., and Sogayar, M. C. (2008) *Genet. Mol. Res.*, **7**, 371-378.
29. Rajkumar, T., Sabitha, K., Vijayalakshmi, N., Shirley, S., Bose, M. V., Gopal, G., and Selvaluxmy, G. (2011) *BMC Cancer*, **11**, 80.
30. Noda, T., Yamamoto, H., Takemasa, I., Yamada, D., Uemura, M., Wada, H., Kobayashi, S., Marubashi, S., Eguchi, H., Tanemura, M., Umeshita, K., Doki, Y., Mori, M., and Nagano, H. (2012) *Liver Int.*, **32**, 110-118.
31. Reis, P. P., Waldron, L., Perez-Ordonez, B., Pintilie, M., Galloni, N. N., Xuan, Y., Cervigne, N. K., Warner, G. C., Makitie, A. A., Simpson, C., Goldstein, D., Brown, D., Gilbert, R., Gullane, P., Irish, J., Jurisica, I., and Kamel-Reid, S. (2011) *BMC Cancer*, **11**, 437.
32. Annunen, P., Helaakoski, T., Myllyharju, J., Veijola, J., Pihlajaniemi, T., and Kivirikko, K. I. (1997) *J. Biol. Chem.*, **272**, 17342-17348.
33. Myllyharju, J., and Kivirikko, K. I. (2004) *Trends Genet.*, **20**, 33-43.
34. Bordoli, M. R., Stiehl, D. P., Borsig, L., Kristiansen, G., Hausladen, S., Schraml, P., Wenger, R. H., and Camenisch, G. (2011) *Oncogene*, **30**, 548-560.
35. Jokilehto, T., Rantanen, K., Luukkaa, M., Heikkinen, P., Grenman, R., Minn, H., Kronqvist, P., and Jaakkola, P. M. (2006) *Clin. Cancer Res.*, **12**, 1080-1087.
36. Wang, G. L., and Semenza, G. L. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 4304-4308.
37. Semenza, G. L. (2007) *Drug Discov. Today*, **12**, 853-859.
38. Ivan, M., Haberberger, T., Gervasi, D. C., Michelson, K. S., Gunzler, V., Kondo, K., Yang, H., Sorokina, I., Conaway, R. C., Conaway, J. W., and Kaelin, W. G., Jr. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 13459-13464.
39. Natarajan, R., Salloum, F. N., Fisher, B. J., Kukreja, R. C., and Fowler, A. A., 3rd (2006) *Circ. Res.*, **98**, 133-140.