Krüppel-like factor 5 in human breast carcinoma: a potent prognostic factor induced by androgens

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Abstract

Krüppel-like factor 5 (intestinal) or Krüppel-like factor 5 (*KLF5*) is a zinc finger-containing transcription factor and involved in important biological processes including cell proliferation and differentiation. However, clinical significance of *KLF5* protein has remained largely unknown in breast cancer. Therefore, in this study, we immunolocalized *KLF5* in 113 human breast carcinoma cases. *KLF5* immunoreactivity was frequently detected in the nuclei of breast carcinoma cells, and median value of the ratio of *KLF5*-positive carcinoma cells was 30% and was positively associated with the status of androgen receptor. *KLF5* immunoreactivity was also significantly associated with increased risk of recurrence and worse clinical outcome in breast cancer patients by univariate analyses, and subsequent multivariate analyses demonstrated that *KLF5* immunoreactivity was an independent prognostic factor for both disease-free and breast cancer-specific survival of the patients. We then examined possible regulation of *KLF5* by androgen using MCF-7 breast carcinoma cells. *KLF5* mRNA was induced by biologically active androgen 5 α -dihydrotestosterone in a dose- and time-dependent manner in MCF-7 cells. In addition, results of transfection experiments demonstrated that proliferation activity of MCF-7 cells was significantly associated with the *KLF5* expression level. These findings suggest that *KLF5* is an androgen-responsive gene in human breast carcinomas and play important roles in the progression of breast carcinomas. *KLF5* immunoreactivity is therefore considered a potent prognostic factor in human breast cancers.

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Introduction

Breast cancer is one of the most common malignancies in women worldwide. Human breast tissue is a target of sex steroids, and both estrogens and androgens play important roles in the development and/or progression of hormone-responsive breast carcinomas.

Krüppel-like factor 5 (intestinal) or Krüppel-like factor 5 (*KLF5*) is a zinc finger-containing transcription factor and ubiquitously expressed in various human tissues such as breast, stomach, and colon (Conkright *et al.* 1999, Shi *et al.* 1999, Chen *et al.* 2002, 2003). *KLF5* is also known to be involved in

important biological processes such as cell proliferation and differentiation (Bieker 2001, Black *et al.* 2001), and its expression is induced by growth stimuli, consistent with its potent pro-proliferating roles (Bieker 2001, Sun *et al.* 2001). Recently, *KLF5* has been proposed to be involved not only in normal human tissues but also in their malignant tumors (Tong *et al.* 2006, Kwak *et al.* 2007, Chen *et al.* 2011, Nakajima *et al.* 2011, Soon *et al.* 2011). However, its biological functions and/or clinical significance in human carcinomas have remained largely unknown, which is partly due to the fact that results of previous reports varied among different types of carcinomas

(reviewed in Dong & Chen (2009)). For instances, Kwak *et al.* (2007) reported that KLF5 immunoreactivity was detected in only 47% of gastric cancer patients, while it was detected in a majority of normal stomach, and demonstrated that KLF5 immunoreactivity was a favorable prognostic factor in gastric cancer. However, Soon *et al.* (2011) recently demonstrated KLF5 as a worse prognostic factor in gastric cancer using immunohistochemistry. KLF5 was also reported to inhibit cell proliferation of esophageal carcinoma cells (Yang *et al.* 2005), but its expression was also reported to be associated with activating *KRAS* mutations and to promote both tumorigenesis and cell proliferation in the colorectal carcinomas (Nandan *et al.* 2008).

In breast cancer, *KLF5* was reported to promote breast carcinoma cell proliferation by inducing fibroblast binding protein 1 (Liu *et al.* 2009, Zheng *et al.* 2009). In addition, Liu *et al.* (2011) reported that KLF5 was involved in progesterone-dependent breast carcinoma cell proliferation. Tong *et al.* (2006) also demonstrated *KLF5* mRNA as a prognostic factor in human breast cancers using 90 breast cancer specimens. However, it is also true that KLF5 protein is rapidly degraded by ubiquitin–proteasome system (Chen *et al.* 2005, Liu *et al.* 2010, Zhao *et al.* 2010), and *KLF5* mRNA level may not always reflect its biological functions. Overexpression of KLF5 protein in breast carcinoma tissues has been reported by Chen *et al.* (2011), but it remains unclear whether KLF5 protein can be a prognostic factor or not in the patients with breast cancer as reported in other human malignancies. Therefore, in this study, we first immunolocalized KLF5 in breast cancer tissues and correlated the findings with various clinicopathological factors to assess clinicopathological significance of KLF5 in breast cancer patients.

Materials and methods

Patients and tissues

One hundred and thirteen specimens of invasive ductal carcinoma of human breast were obtained from women who underwent surgical treatment from 1990 to 1999 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. All the patients did not receive chemotherapy, irradiation, or hormonal therapy before the surgery. The clinical outcome of the patients was evaluated by both disease-free and breast cancer-specific survival. The mean age was 56 years (range 31–81 years), and the mean follow-up time was 103 months (range 1–175 months). All the specimens had been fixed in 10% formalin and embedded in paraffin

wax. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (approval number 2008-288).

Immunohistochemistry

Characteristics of primary antibody for KLF5 were reported previously (Kwak *et al.* 2007). Mouse monoclonal antibodies for estrogen receptor (ER; ER1D5), progesterone receptor (PR; MAB429), androgen receptor (AR; AR441), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA) respectively. Rabbit polyclonal antibody for HER2 (A0485) was purchased from DAKO. A Histofine Kit (Nichirei, Tokyo, Japan), which employs streptavidin–biotin amplification method, was used. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine and counterstained with hematoxylin. Human tissue of the stomach was used as a positive control for KLF5 antibody (Kwak *et al.* 2007), and PBS was used instead of the primary antibody as a negative control of KLF5 immunostaining in this study.

Scoring of immunoreactivity and statistical analyses

KLF5, ER, PR, AR, and Ki-67 immunoreactivity was detected in the nucleus of breast carcinoma cells, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case, and subsequently, the percentage of immunoreactivity, i.e. labeling index (LI), was determined. KLF5 immunoreactivity was subsequently classified into two groups (i.e. high and low groups) according to the median value of KLF5 LI (Ferreira *et al.* 2001). Cases with ER LI of more than 1% were considered ER-positive breast carcinoma, according to a previous report (Hammond *et al.* 2010). HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and strongly circumscribed membrane staining of HER2 in more than 10% carcinoma cells was considered positive in this study.

An association between KLF5 immunoreactivity and clinicopathological factors was evaluated using one-way ANOVA and Bonferroni test or a cross-table using χ^2 test. Breast cancer-specific and disease-free survival curves were generated according to Kaplan–Meier method; statistical significance was calculated using log-rank test. Univariate and multivariate analyses were evaluated by proportional hazard model (COX). *P* values <0.05 were considered significant in this study.

Cell line and chemicals

Human breast cancer cell line MCF-7 was provided from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan), and cultured in RPMI 1640 (Sigma–Aldrich) with 10% fetal bovine serum (FBS; FJRH Bioscience, Lenexa, KS, USA). MCF-7 cells were cultured in phenol-red-free RPMI 1640 with 10% dextran-coated charcoal-stripped FBS for 3 days before the treatment of sex steroids.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen). Real-time PCR was performed using the LightCycler System and FastStart DNA Master SYBR Green I (Roche Diagnostics). The primer sequence of KLF5 and the ribosomal protein L13A (RPL13A) was as follows. KLF5: forward 5'-ATAGAAGGAGTAACCCCGAT-3' and reverse 5'-CACTTGTATGGCTTTTCACC-3'; and RPL13A: forward 5'-CCTGGAGGA-GAAGAG-GAAAGAGA-3' and reverse 5'-TTGAGGACC-TCTGTGTATTTGTCAA-3' (Takagi *et al.* 2010). KLF5 mRNA level was calculated as the ratio of RPL13A mRNA level.

Luciferase assay

In this study, two androgen-responsive reporter plasmids were used (i.e. pKLF5-ARE-Luc and pPSAE-Luc plasmids). At first, pKLF5-ARE-Luc plasmids were constructed to examine possible recruitment of AR into putative androgen-responsive element(s) (ARE) in first intron of *KLF5* gene identified by Frigo *et al.* (2009). Briefly, 158 bp DNA region containing ARE within was amplified by PCR method using adapter primers (forward 5'-TAGGTACCTCAGTGGATACAAGGTTTTGC-3' and reverse 5'-TATAAGCTTCGATGCAAACCTT-TAAAATATTGC-3'). DNA fragment was digested with HindIII and KpnI and cloned into pGL3 basic luciferase reporter vectors (Promega). Secondly, we used pPSAE-Luc, androgen-responsive reporters,

Figure 1 Immunohistochemistry for KLF5 in the breast carcinoma. (A and B) Representative picture of KLF5-high (A) and KLF5-low (B) cases. KLF5 immunoreactivity was detected in the nucleus of carcinoma cells. AR LIs in each case was 56% (A) and 2% (B) respectively. (C) KLF5 immunoreactivity was focally detected in the nucleus of epithelium in the morphologically normal mammary glands. (D) Positive control (left) and negative control (right) section of KLF5 immunohistochemistry (fundic glands of the stomach). Bar = 100 μ m respectively. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-12-0017>.

which contained ARE of KLK3 (Takagi et al. 2010) in order to examine transcriptional activity of AR.

One microgram of pKLF5-ARE-Luc or pPSAE-Luc and 200 ng pRL-TK-control plasmids (Promega) were used to measure the transcriptional activity of endogenous AR. Transient transfections were carried out using TransIT-LT Transfection Reagents (TaKaRa, Tokyo, Japan) in MCF-7 cells, and the luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and luminescencer-PSN (AB-2200; Atto Co., Tokyo, Japan) after incubation with 10 nM 5 α -dihydrotestosterone (DHT) for 24 h. The transfection efficiency was normalized against Renilla luciferase activity using pRL-KT control plasmids, and the luciferase activity for each sample was evaluated as the ratio (%) compared with that of controls.

Small interfering RNA transfection

Small interfering RNA (siRNA) oligonucleotides for KLF5 (siKLF5) were obtained from Genolution Pharmaceuticals, Inc. (Seoul, South Korea). The target sequences of siKLF5 were as follows: sense 5'-GUCG-UAGACCAGUUCUUCAUU-3' and antisense 5'-UG-AAGAACUGGUCUACGACUU-3'. In addition, medium GC duplex #2 (Invitrogen) was used as a negative control (siCTRL). The siRNA was transfected using HiperFect transfection reagent (Qiagen).

Cell proliferation assay

MCF-7 cells were transfected with siKLF5 or siCTRL in a 96-well culture plate. Three days after transfection, the cell proliferation was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Results

Immunolocalization of KLF5 in human breast carcinomas

KLF5 immunoreactivity was detected in the nuclei of carcinoma cells (Fig. 1A and B), and the median LI of KLF5 was 30% (range 2–99%). KLF5 immunoreactivity was focally detected in the epithelial cells of morphologically normal mammary glands, but its LI (ranged from 0 to 15%) was much lower than that in carcinoma cells (Fig. 1C). In the positive control, KLF5 immunoreactivity was detected in fundic glands of the normal stomach (left panel of Fig. 1D) as reported previously (Kwak et al. 2007), whereas no significant immunoreactivity was detected

in the same area of negative control section (right panel of Fig. 1D).

KLF5 immunoreactivity was then classified into two groups according to median KLF5 LI (30%), and cases with KLF5 LI of more than 30% were considered KLF5-high breast carcinomas in this study. Associations between KLF5 immunoreactivity and various clinicopathological parameters in 113 breast carcinomas were summarized in Table 1. Significant correlation was detected between KLF5 immunoreactivity and AR LI ($P=0.039$), while no significant associations were detected between KLF5 immunoreactivity and other parameters examined. Significant correlation between KLF5 immunoreactivity and AR LI was also detected when KLF5 LI was used as a continuous variable ($P=0.038$ and $r=0.20$, data not shown).

Table 1 Association between KLF5 immunoreactivity and clinicopathological parameters in 113 breast carcinomas

	KLF5 immunoreactivity		P value
	High (n=57)	Low (n=56)	
Age (years) ^a	55.5±3.1	57.2±3.1	0.43
Menopausal status			
Premenopausal	21 (19%)	17 (15%)	0.28
Postmenopausal	36 (32%)	39 (35%)	
Stage			
I+II	45	42	0.78
III+IV	12	14	
Pathological tumor factor (pT)			
pT1	19 (17%)	25 (22%)	0.30
pT2–4	38 (34%)	31 (27%)	
Lymph node status			
Positive	30 (27%)	21 (19%)	0.15
Negative	27 (24%)	35 (31%)	
Distant metastasis			
Positive	6 (5%)	2 (2%)	0.28
Negative	51 (45%)	54 (48%)	
Histological grade			
1, 2 (well or moderate)	40	39	>0.99
3 (poor)	17	17	
ER status			
Positive	49 (43%)	43 (38%)	0.31
Negative	8 (7%)	13 (12%)	
ER LI (%) ^a	48.8±9.5	47.4±10.0	0.84
PR LI (%) ^a	30.4±8.4	28.2±8.1	0.71
AR LI (%) ^a	34.2±7.5	24.3±5.8	0.039
HER2 status			
Positive	13 (12%)	8 (7%)	0.36
Negative	44 (39%)	48 (42%)	
Ki-67 LI (%) ^a	19.7±3.9	20.2±4.7	0.86

P value <0.05 were considered significant, and described as boldface.
^aData was presented as mean±95% confidence interval (95% CI) or the number of cases with percentage.

Correlation between KLF5 immunoreactivity and clinical outcome of breast cancer patients

In order to examine the association between KLF5 immunoreactivity and patient prognosis, we excluded stage IV cases and used stage I–III breast carcinoma patients ($n=105$) in the following analyses. As demonstrated in Fig. 2A, KLF5 immunoreactivity was significantly associated with increased risk of recurrence ($P=0.033$ by log-rank test), and multivariate analysis revealed that lymph node metastasis ($P=0.0049$), pathological tumor factor (pT) ($P=0.0498$), and KLF5 immunoreactivity ($P=0.0095$) were independent prognostic factors for disease-free survival with relative risks over 1.0 (Table 2).

Association between KLF5 immunoreactivity and breast cancer-specific survival was summarized in Fig. 2B. Significant association was detected between KLF5 immunoreactivity and adverse clinical outcome in 105 breast carcinoma patients examined ($P=0.014$ by log-rank test). Multivariate analysis demonstrated that only KLF5 immunoreactivity ($P=0.031$) was an independent prognostic factor with relative risk over 1.0 (Table 3). Similar tendency was also detected when KLF5 immunoreactivity was further divided into four groups ($P=0.032$ for disease-free survival (Fig. 2C) and P value was not calculated for breast cancer-specific survival because no patients died in the group with KLF5 LI of $<10\%$ (Fig. 2D)). When KLF5 LI was used as a continuous variable, it was also indicated as an independent prognostic factor for both disease-free and breast cancer-specific survival ($P=0.0045$ and $P=0.0050$ respectively) (data not shown).

KLF5 as an androgen-responsive gene in breast carcinomas

We detected a significant correlation between KLF5 and AR in our immunohistochemical analysis as shown in Table 1, which suggested a possible regulation of KLF5 by androgens in human breast carcinoma cells.

Figure 2 Disease-free and breast cancer-specific survival of 105 breast carcinoma patients according to KLF5 immunoreactivity. (A and B) KLF5 immunoreactivity was significantly associated with an increased risk of recurrence ($P=0.033$) (A) and worse prognosis ($P=0.014$) (B). Solid line, high-KLF5 immunoreactivity ($n=51$); and dashed line, low-KLF5 immunoreactivity ($n=54$). (C and D) Disease-free (C) and breast cancer-specific (D) survival curves when KLF5 immunoreactivity was further divided into four groups (i.e. 0–9% (dotted line), 10–29% (dashed line), 30–50% (dot-dashed line), and $\geq 50\%$ (solid line) of KLF5 LI). P values were evaluated by log-rank test. In D, P value was not calculated because no patients died in the group with KLF5 LI of $<10\%$.

Table 2 Univariate and multivariate analyses of disease-free survival in stages I–III breast cancer patients examined (*n*=105)

Variable	Univariate	Multivariate	
	<i>P</i>	<i>P</i>	Relative risk (95% CI)
Lymph node status (positive/negative)	0.0059	0.0049	4.51 (1.58–12.9)
KLF5 immunoreactivity (high/low)	0.0075	0.0095	4.42 (1.44–13.4)
HER2 status (positive/negative)	0.037	0.056	
pT (2, 3/1)	0.013	0.0498	3.51 (1.00–12.3)
Ki-67 LI (82-0%)	0.55		
ER status (negative/positive)	0.64		
Histological grade (3/1, 2)	0.95		

Data considered significant (*P*<0.05) in the univariate analyses were described as boldface, and these parameters were examined in the multivariate analyses.

However, such findings have not been reported yet to the best of our knowledge. Therefore, we performed following *in vitro* experiments using MCF-7 cells, which expressed both ER and AR (Horwitz *et al.* 1975).

As demonstrated in Fig. 3A, *KLF5* mRNA was significantly induced by bioactive androgen DHT in a dose-dependent manner, and this increment became significant from 10 pM (1.8-fold, *P*<0.05). However, DHT-mediated induction of *KLF5* mRNA was significantly suppressed to the basal level by addition of AR antagonist bicalutamide (0.35-fold compared with DHT (10 nM) alone, *P*<0.0001). Bicalutamide alone did not significantly (0.96-fold, *P*=0.71) change *KLF5* mRNA levels in MCF-7 cells. DHT (10 nM) also induced *KLF5* mRNA in MCF-7 cells in a time-dependent fashion (Fig. 3B), and the induction became significant after 8 h from DHT treatment (1.7-fold, *P*<0.05). Similar results were obtained when other breast carcinoma cell line T-47D, which expressed both ER and AR (Migliaccio *et al.* 2000), was used (data not shown). On the other hand, estradiol did not significantly influence *KLF5* mRNA expression in MCF-7 cells (1.2-fold, *P*=0.51; data not shown).

In order to examine whether *KLF5* was a primary responsive gene of androgen or not, MCF-7 cells were treated together with DHT and protein synthesis inhibitor cycloheximide. As demonstrated in Fig. 3C, cycloheximide did not completely suppress DHT-mediated induction of *KLF5* (2.5-fold, *P*<0.001, compared with control group).

We next performed luciferase assay to examine possible recruitment of AR into ARE of *KLF5* gene previously identified by Frigo *et al.* (2009) using pKLF5-ARE-Luc plasmids. The luciferase activity was not significantly increased by DHT (0.99-fold, *P*=0.93) in MCF-7 cells, although it was 1.2-fold (*P*=0.04) increased in T-47D cells (Fig. 3D).

Effects of KLF5 on breast cancer cell proliferation and AR function

The results of our study suggested that *KLF5* was associated with worse prognosis of breast carcinoma patients (Fig. 2). In order to further examine the effects of *KLF5* on breast cancer cell proliferation, we transfected specific siRNA for *KLF5* (siKLF5) in MCF-7 cells. *KLF5* mRNA level was markedly (0.54-fold, *P*<0.001) suppressed in the cells transfected with siKLF5 compared with those transfected with nonspecific control siRNA (siCTRL) at the 3 days after the transfection (Fig. 4A). As shown in Fig. 4B, cell proliferation activity was significantly suppressed in the cells transfected with siKLF5 compared with those transected with siCTRL (0.67-fold, *P*<0.05).

We also found out a positive association between *KLF5* and AR immunoreactivity in breast carcinoma tissues (Table 1) and upregulation of *KLF5* mRNA expression by androgens. Therefore, it may be possible to speculate the modulation of AR function by *KLF5*. To assess this hypothesis, we performed luciferase assay using ARE reporter plasmids pPSAE-luc. However, DHT-mediated luciferase activity of MCF-7 cells was not significantly (*P*=0.86) changed between siCTRL and siKLF5 groups (Fig. 4C).

Table 3 Univariate and multivariate analyses of breast cancer-specific survival in stage I–III breast cancer patients examined (*n*=105)

Variable	Univariate	Multivariate	
	<i>P</i>	<i>P</i>	Relative risk (95% CI)
Lymph node status (positive/negative)	0.15		
KLF5 immunoreactivity (high/low)	0.042	0.031	11.5 (1.24–107)
HER2 status (positive/negative)	0.0042	0.32	
pT (2, 3/1)	0.074		
Ki-67 LI (82-0%)	0.0097	0.60	
ER status (negative/positive)	0.019	0.41	
Histological grade (3/1, 2)	0.0046	0.14	

Data considered significant (*P*<0.05) in the univariate analyses were described as boldface, and these were analyzed in the multivariate analyses.

Discussion

This is the first study that demonstrated the association between KLF5 immunoreactivity and clinical outcome of breast carcinomas to the best of our knowledge. KLF5 immunoreactivity was detected in 50% of breast

cancer cases and was increased compared with morphologically normal mammary glands. KLF5 immunoreactivity was also significantly associated with increased risk of recurrence and poor prognosis in breast cancer patients, and subsequent multivariate analyses revealed that KLF5 immunoreactivity was an independent prognostic factor for both recurrence and breast cancer-specific survival of the patients. These findings are in good agreement with the previous report by Tong *et al.* (2006), which demonstrated significant association between *KLF5* mRNA level and poor prognosis of breast cancer patients. Results of subsequent *in vitro* experiments demonstrated that KLF5 significantly promoted breast cancer cell proliferation. These findings indicated possible roles of KLF5 in the progress of breast carcinomas. In prostate carcinomas, however, KLF5 is considered a better prognostic marker (Nakajima *et al.* 2011) and expression of KLF5 was also reported to be suppressed in prostate cancer (Chen *et al.* 2003). These differences among different types of human malignancies may be partly explained by diversity of the expression patterns of *KLF5* responsive genes because KLF5 is known to regulate several different genes associated with cell proliferation (reviewed in Dong & Chen (2009)). Bateman *et al.* (2004) reported that KLF5 mediated cyclin D1 expression in untransformed intestinal epithelial cells, while cyclin D1 was also considered a poor prognostic factor in breast carcinomas (Kenny *et al.* 1999). KLF5 was also reported to be essential for the expression of c-myc (Guo *et al.* 2009), which was considered poor prognostic factor in breast cancer patients (Berns *et al.* 1992). Therefore, *KLF5* may contribute to the progression of breast carcinomas by regulating genes associated with poor prognosis of breast cancer patients but further examinations of *KLF5* responsive genes are required to clarify the molecular mechanisms of KLF5 in the breast carcinoma or its tissue-specific functions.

Figure 3 Induction of *KLF5* expression by DHT in MCF-7 breast carcinoma cells. (A, B and C) Effects of DHT on *KLF5* mRNA expression were examined by real-time PCR analysis. MCF-7 cells were treated with indicated concentrations of DHT with or without an AR blocker bicalutamide (BIC) (A) or cycloheximide (CHX) (C) for 72 h, treated with DHT (10 nM) for indicated period (B). *KLF5* mRNA level was evaluated as a ratio of *RPL13A* mRNA level, and subsequently relative *KLF5* mRNA level was summarized a ratio (%) compared with the basal level (nontreatment). (D) Recruitment of AR into ARE was examined by luciferase assay. MCF-7 and T-47D cells were treated with DHT (10 nM) for 24 h. Relative luciferase activity was evaluated as a ratio (%) of basal level (nontreatment). Data were presented as the mean $\pm$ s.d. ($n=3$) respectively. * $P < 0.05$, *** $P < 0.001$ vs nontreatment (left column), and ††† $P < 0.001$ vs DHT (10 nM)-treated group. The statistical analyses were performed using one-way ANOVA and Bonferroni tests.

Figure 4 Effects of *KLF5* on breast carcinoma cell proliferation and AR function. (A) Knockdown of *KLF5* mRNA level in MCF-7 cells transfected with specific siRNA for *KLF5* (siKLF5) compared with those transfected with nonspecific control siRNA (siCTRL) was examined by real-time PCR analysis. *KLF5* mRNA level was evaluated as a ratio of *RPL13A* mRNA level, and subsequently, relative *KLF5* mRNA level was summarized a ratio (%) compared with siCTRL group. (B) Relative cell proliferation was evaluated as a ratio (%) compared with siCTRL group. (C) DHT-dependent transactivation was examined by luciferase assay. Relative luciferase activity was evaluated as a ratio (%) compared with siCTRL group. Data were presented as the mean $\pm$ s.d. ($n=3$) respectively. * $P<0.05$ and *** $P<0.001$ vs nontreatment (left column). The statistical analyses were performed using one-way ANOVA and Bonferroni tests.

In our present study, the status of *KLF5* immunoreactivity was significantly correlated with AR LI. Therefore, we examined possible regulation of *KLF5* by androgen using *in vitro* studies. Real-time PCR

analyses revealed that *KLF5* mRNA was significantly induced by DHT in both dose- and time-dependent manners. Moreover, cycloheximide did not affect DHT-mediated induction of *KLF5* mRNA level, suggesting that *KLF5* was a primary responsive gene of androgen. In consistent with our present results Frigo *et al.* (2009), previously reported a direct induction of *KLF5* mRNA expression by R1881, synthetic androgen, in a prostate cancer cell line (LNCaP). They also identified putative ARE in first intron of *KLF5* gene. However, subsequent luciferase assay revealed that this ARE was not necessarily activated by endogenous AR in the breast carcinoma cells in our study. As five potential AREs were found in -4 kbp upstream region of *KLF5* gene (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>), *KLF5* expression may be regulated by androgen through several AREs in the promoter region in the breast carcinoma cells. In addition, it is also true that Liu *et al.* (2011) reported an induction of *KLF5* by progesterone in breast cancer cell line (T-47D). In our present study, 25% of high *KLF5* immunoreactivity breast cancers (14 out of 57 cases) were associated with negative AR status (i.e. AR LI $<10\%$) (data not shown). Therefore, *KLF5* expression may be regulated by several factors other than androgen and may not necessarily reflect the sequels of androgen actions in the breast carcinomas.

Pro-proliferating roles of androgens have been recently reported by several groups, although androgens are generally considered to exert antiproliferative effects in breast carcinomas (reviewed in Suzuki *et al.* (2010)). Especially, functional cross talk between AR and HER2 signaling has been proposed in ER-negative/HER2-positive breast carcinomas (Naderi & Hughes-Davies 2008, Ni *et al.* 2011). Naderi & Hughes-Davies (2008) demonstrated that growth stimulatory effect of heregulin, a ligand of HER1 and HER3, was inhibited by AR inhibitor flutamide in MDA-MB-453 cells. On the other hand, Gregory *et al.* (2005) demonstrated that heregulin-induced activation of HER2 and HER3 increased AR transactivation and Beckers *et al.* (2005) previously reported that overexpression of HER2 induced *KLF5* expression using microarray analysis. Taken together with these previous findings and our present results, pro-proliferating effects of androgens may be, at least in a part, mediated by induction of *KLF5* through AR in the breast carcinoma. Multicenter phase II trial of bicalutamide for ER-negative/AR-positive metastatic breast cancer are ongoing (NCT00468715), and our present study may serve as a starting point for clarification of molecular mechanisms and/or predictive marker of AR-based therapy in the breast carcinoma patients.

In summary, KLF5 immunoreactivity was frequently detected in breast cancer tissues, and its immunoreactivity was positively associated with AR LI. KLF5 immunoreactivity was significantly associated with increased risk of recurrence and worse prognosis of breast carcinoma patients. Subsequent *in vitro* studies revealed that *KLF5* mRNA was induced by DHT and *KLF5* expression level was associated with the proliferation activity in MCF-7 cells. These results suggested that *KLF5* was an androgen-responsive gene and plays important roles in the progression of breast carcinomas.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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