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Gui-Shan Jin[*] Eisaku Kondo[†] Takayoshi Miyake[‡]

Masao Shibata[**] Takako Takashima[††] Yi-Xuan Liu[‡‡]

Kazuhiko Hayashi[§] Tadaatsu Akagi[¶] Tadashi Yoshino[‖]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Medical and Biological Laboratories Co. Ltd.,,
[††]Medical and Biological Laboratories Co. Ltd.,,
[‡‡]Okayama University,
[§]Okayama University,
[¶]Okayama University,
[‖]Okayama University,

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Abstract

FKHRL1 (FOXO3a), a member of the Forkhead family of genes, has been considered to be involved in the development of breast tumors; however, the in vivo expression and activation status of FKHRL1 in breast tumors still remains unclear. We immunohistochemically demonstrated the expression and intracellular localization of FKHRL1 in human breast tumors by the novel anti-FKHRL1 antibody which is available for formalin-fixed paraffin-embedded specimens. In a total of 51 cases of benign tumors, FKHRL1 was diffusely expressed in all cases, and its intracellular localization was revealed to be cytoplasmic (inactive form) in 94% of cases of intraductal papillomas (16/17) and 91% cases of fibroadenomas (31/34), with a similar pattern to normal glandular epithelium. In invasive ductal carcinomas, 83% of the cases (93/112) diffusely expressed FKHRL1; however, unlike benign tumors, 71% of the cases (66/93) showed the nuclear-targeted, active form of FKHRL1. Moreover, activated FKHRL1 was predominantly observed in scirrhous (29/36, 81% of the cases) and papillotubular (30/38, 79% of the cases) subtypes, compared to the solid-tubular subtype (7/19, 37% of the cases). Furthermore, the cases with nuclear-targeted FKHRL1 showed a tendency to have lymph nodal metastasis with statistical significance ($P < 0.0001$). Thus, the activation of FKHRL1 seems to be recognized as one of the specific features of invasive ductal carcinoma of the breast.

KEYWORDS: FKHRL1, intracellular localization, breast tumors

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Original Article

Expression and Intracellular Localization of FKHRL1 in Mammary Gland Neoplasms

Gui-Shan Jin[a], Eisaku Kondo[a]*, Takayoshi Miyake[a], Masao Shibata[b], Takako Takashima[b], Yi-Xuan Liu[a], Kazuhiko Hayashi[a], Tadaatsu Akagi[a], and Tadashi Yoshino[a]

[a]Department of Pathology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan, and [b]Ina Institute, Medical and Biological Laboratories Co. Ltd., Nagano 396-0002, Japan

FKHRL1 (FOXO3a), a member of the Forkhead family of genes, has been considered to be involved in the development of breast tumors; however, the *in vivo* expression and activation status of FKHRL1 in breast tumors still remains unclear. We immunohistochemically demonstrated the expression and intracellular localization of FKHRL1 in human breast tumors by the novel anti-FKHRL1 antibody which is available for formalin-fixed paraffin-embedded specimens. In a total of 51 cases of benign tumors, FKHRL1 was diffusely expressed in all cases, and its intracellular localization was revealed to be cytoplasmic (inactive form) in 94% of cases of intraductal papillomas (16/17) and 91% cases of fibroadenomas (31/34), with a similar pattern to normal glandular epithelium. In invasive ductal carcinomas, 83% of the cases (93/112) diffusely expressed FKHRL1; however, unlike benign tumors, 71% of the cases (66/93) showed the nuclear-targeted, active form of FKHRL1. Moreover, activated FKHRL1 was predominantly observed in scirrhous (29/36, 81% of the cases) and papillotubular (30/38, 79% of the cases) subtypes, compared to the solid-tubular subtype (7/19, 37% of the cases). Furthermore, the cases with nuclear-targeted FKHRL1 showed a tendency to have lymph nodal metastasis with statistical significance ($P < 0.0001$). Thus, the activation of FKHRL1 seems to be recognized as one of the specific features of invasive ductal carcinoma of the breast.

Key words: FKHRL1, intracellular localization, breast tumors

The forkhead family of transcription factors is a large group of proteins that share a common conserved 100 amino acid DNA binding domain, which is termed a winged-helix or forkhead domain after the founding member of this group, the forkhead gene in *Drosophila* [1]. In addition to their roles during normal development, recent studies have demonstrated that members of the forkhead family may participate in several kinds of neoplasia [2-4]. In particular, 3 genes of the human forkhead family, FKHR, FKHRL1, and AFX, which are orthologues of DAF-16 in *Caenorhabditis elegans*, were identified at the chromosomal breakpoints in human neoplasms [5-7]. These three genes have been assigned to the FOXO (named for "Forkhead Box, group O") subfamily of forkhead transcription factors following the updated nomenclature [8].

Previous studies have also revealed that the forkhead factors, including FKHRL1, regulate their activation through phosphorylation by proto-oncogene Akt (protein kinase B) and/or SGK (serum- and glucocorticoid-

Received February 5, 2004; accepted March 8, 2004.
*Corresponding author. Phone: +81-86-235-7151; Fax: +81-86-235-7156
E-mail: ekondo@md.okayama-u.ac.jp (E. Kondo)

induced kinases) in mammalian cells [9–11], and the phosphorylated form localized at cytoplasm is recognized to be an inactivated form, whereas nuclear-imported dephosphorylated FKHRL1 is functional as an active transcriptional factor [9, 10]. Previous studies reported that FKHRL1 activation induced apoptosis by recruiting the Fas ligand (FasL), and, therefore, activation of Akt resulted in cell survival by phosphorylating FKHRL1, which disrupted the apoptotic function of FKHRL1 [9]. However, recent accumulating data provides information on novel aspects of FKRHL1, notably the fact that it is regulated not only by Akt, but other molecules such as nitric oxide-ROCK (in breast carcinoma cell lines), FSH-IGF-I (in ovarian follicle cells), and p66shc (in neuronal cells) [9, 10, 12–14]. Furthermore, in addition to FasL, it was reported that activated FKHRL1 can induce various kinds of genes, such as p27kip1, the p130 RB family protein, the Bim of Bcl2 family protein, and TGF-β at its downstream [15–19]. Thus, FKHRL1 seems to be involved in complicated intracellular signalings, and its total functional aspect has not yet been fully disclosed. Taking this situation into consideration, information about the *in vivo* expression of FKHRL1 in human neoplasms is considered to be important, since it directly reflects the functional status of FKHRL1 on tumor cells *in vivo*. However, little is known about it, because previous studies were largely restricted to transfection experiments using established cell lines, including breast carcinomas.

Here we report on the endogenous expression and intracellular localization of FKHRL1, which reflect its activation status in surgically-obtained mammary neoplasmic tissues from patients, and also attempt to explain its biological significance *in vivo*.

Materials and Methods

Tissues samples. 163 cases of mammary gland tumors of the breast, consisting of 17 cases of intraductal papilloma with a mean age of 35 years, 34 cases of fibroadenoma with a mean age of 47 years, and 112 cases of invasive ductal carcinoma with a mean age of 57 years were randomly collected from the files of the Department of Pathology, University Hospital Okayama Graduate School of Medicine and Dentistry, from 2000–2003. All of the patients were women. In the invasive ductal carcinomas, 45 cases were the papillotubular type, 43 cases were the scirrhous type, 24 cases were the solid-tubular type (according to the histological classification of "General Rules for Clinical and Pathological Recording of Breast Cancer" published by the Japanese Breast Cancer Society, 14 th Edition). Among the 112 cases of invasive ductal carcinomas, 37 cases accompanied with lymph node metastasis were observed among the 94 cases in which the lymph node data were available. Haematoxylin-eosin (HE)-stained sections were re-evaluated to confirm the tumor histological type. All cases were surgically resected breast tissue specimens. None of the patients of the selected cases in this study underwent radiation or chemotherapy prior to surgery. All of the surgical materials were used in the present study by obtaining the infomed consent of the patients.

Generation of novel anti-FKHRL1 polyclonal antibody. The N-terminal region of human FKHRL1 cDNA fragment encoding 131 amino acids was cloned from cDNA of the human B-cell lymphoma cell line, BALM-14 [20, 21], by employing the RT-PCR method. The primers' sequences for RT-PCR were as follows: forward primer; 5'-GAATTCATGGCAGAG GCACCG-3', reverse primer; 5'-CTCGAGTTGCTG AGGCTGCAG-3'. The amplified cDNA fragment was subcloned into pGEX-5X-1 (Amersham Pharmacia, Biotech) at the EcoRI-Xho sites, and expressed in *E. coli*, and the lysates were column-purified to extract GST-fusioned protein by the conventional method. The GST-FKHRL1 fusion protein that was obtained was utilized for immunizing rabbits (boosted). Serum was then collected after checking its titer by ELISA, and examined to see if it was available for immunoblotting and immunohistochemistry. The specificity of the generated antibody was examined by several assays, as described below and in the Results section.

Immunoblotting, cell lines and transfection assays. Immunoblotting analysis and transfected studies were performed to evaluate the specificity of the FKHRL1 polyclonal antibody. To obtain the exogenous expression of FKHRL1, pcDNA3-myc-epitope-tagged FKHRL1 was constructed and subcloned into BHK cells. Before and after treatment with LY294002 (20 μM, the inhibitor of PI3K, Calbiochem, CA, USA), whole-cell extracts were resolved by SDS-PAGE and immunoblotted with the $\times$ 50,000 rabbit anti-human FKHRL1 polyclonal antibody and $\times$ 50,000 anti-myc-epitope tagged mouse monoclonal antibody (mAb, 9E10, American Type Culture Collection; ATCC), respectively. In the present study, western blotting was performed by du-

plicating the lanes using the same lysate, which was blotted on the same membrane, divided into 2 pieces, then probed with each antibody, as shown in Fig. 1A. For immunofluorescent assay, the pcDNA3-myc FKHRL1 transfected BHK cell line and breast cancer cell line T47D were used.

Immunohistochemical staining. FKHRL1 immunohistochemical staining was performed according to the following protocol. Four-μm consecutive sections were cut from paraffin-embedded tissue blocks and mounted onto slides coated with 3-aminopropyl-triethoxysilane, and were then deparaffinized and rehydrated with xylene and graded alcohol. The endogenous peroxidase activity was quenched by 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was performed by microwaving the slides in 0.01 mol/L citrate buffer (pH 6.0) within a pressure cooker at 600 w of power for 10 min, followed by cooling at room temperature. The slides were then treated for non-specific binding with 10% normal goat serum in phosphate-buffered saline (PBS) for 15 min, and then incubated overnight at 4 ˚C with an optimal dilution (1:20,000) of a primary polyclonal antibody against human FKHRL1. Sequentially, the slides were detected with the EnVision anti-rabbit kit (DAKO, Carpinteria, CA, USA) using 3,3'-diaminobenzidine, tetrahydrochloride (DAB) as a substrate. Finally, the slides were counterstained for nuclei with Mayer's haematoxylin. The slides were processed in parallel, but with the normal rabbit serum instead of the primary antibody as a negative control in each experiment.

The expression of the phosphorylated form of Akt (P-Akt) was evaluated by using rabbit anti-phospho-Akt, ser473 polyclonal antibody (IHC specific, New England Biolabs) and performed as described above by using P-Akt polyclonal antibody instead of FKHRL1 polyclonal antibody. The antigen retrieval was performed by heating the slides with a microwave at 600 w of power for 1 min instead of 10 min.

Assessment of immunohistochemical staining. The immunohistochemical stainings were scored on 4-μm immune-staining slides using a conventional light microscope at a 40× magnification. FKHRL1 was evaluated to be positive if at least 20% of the tumor cells were apparently stained. For all the cases that showed a positive staining for FKHRL1, the intracellular localization of FKHRL1 was categorized as either nuclear-positive (nuclear alone, or predominantly nuclear, even having both nuclear and cytoplasmic signals), or nuclear-negative (cytoplasmic only), to examine the activation status of endogenous FKHRL1.

The expression of P-Akt was also examined in 10 selected FKHRL1-positive cases (5 nuclear-positive cases, and 5 nuclear-negative cases) to confirm if the FKHRL1-localization correlated to the activation of Akt or not. For each case, the same area of the specimen was evaluated for a comparison.

Statistical analysis. Differences among the FKHRL1 expression patterns, the relationship between the intracellular localization of FKHRL1 and the lymph node metastatic status, and the P-Akt expression were estimated by the chi-square test or Fisher's exact test. A P value of less than 0.05 was considered statistically significant.

Results

Generation of novel anti-FKHRL1 antibody available to paraffin-sectioning. In immunoblot analyses for BHK cells transfected with myc-epitope-tagged full-length human FKHRL1, the newly generated antibody could detect a strong band of approximately 100 kDa in molecular size, which comigrated to the band detected by the anti-myc-epitope-tagged mouse monoclonal antibody (9E10) with or without Ly294002 (Fig. 1A). To confirm whether this anti-FKHRL1 antibody can be used for immunostaining, immunofluoresence assays were also perfomed using this antibody. Ly294002 can inactivate Akt by inhibiting PI3 kinase activity, and it prevented the phosphorylation of FKHRL1 because FKHRL1 is one of the specific substrates of Akt. Intracellularly, FKHRL1 will be imported to the nucleus when its phosphorylation is prevented by the inactivation of Akt. Double immunofluorescence of the transfected BHK cells expressing the myc-epitope-tagged FKHRL1 revealed that the signals detected by this antibody (Cy3-labeled) colocalized with that by the 9E10 (FITC-labeled) well in normal culture conditions, and the antibody also detected nuclear-imported FKHRL1 (*i.e.*, the active form of FKHRL1) in response to treatment with Ly294002 as well as 9E10 did (Fig. 1B). We further examined whether this novel antibody could detect endogenous FKHRL1 by employing the human breast carcinoma cell line, T47D. The endogenous FKHRL1 expression of the T47D cell line was examined by immunoblotting using this antibody prior to immunohisto-

chemical analysis (data not shown). As shown in immunofluorescence images, the antibody recognized both the cytoplasmic (inactive) form and nuclear-imported (active) form of FKHRL1 in response to Ly294002 (Fig. 1 C). The different intracellular localizations of FKHRL1 probed with this antibody were also successfully detected on breast carcinoma cells of paraffin-embedded specimens derived from patients by conventional immunohistochemistry (Fig. 1D). No obvious signal was observed when the antibody was absorbed by 10-folds amount of the original GST-fusioned N-terminal FKHRL1 protein prior to immunohistochemical stainings (data not shown).

Immunohistochemical demonstration of FKHRL1 in normal and neoplastic mammary gland tissues. First, the endogenous expression of FKHRL1 was observed in all of the cases of non-neoplastic mammary glands and ducts, benign mammary gland tumors, and over 80% of invasive ductal carcinomas employed in this study (Table 1).

In non-neoplastic mammary glands and ducts, endogenous FKHRL1 was expressed predominantly at cytoplasms of the glandular epithelium (Fig. 2A, a-d). Benign tumors, including both intraductal papillomas and fibroadenomas, also showed a diffuse cytoplasmic expression pattern of FKHRL1 on tumor cells (Fig. 2A, e-h). In contrast to the benign tumors, invasive ductal carcinoma of the breast showed a predominant nucleus staining among the FKHRL1-positive cases, and the frequency of nuclear-targeted FKHRL1 on the tumor cells was remarkably higher than in the benign breast tumors (Table 2).

Among the invasive ductal carcinoma cases of the breast, 7 cases of papillotubular, 7 cases of scirrhous, and 5 cases of solid-tubular type were FKHRL1 negative. In order to focus on investigating the correlation of the intracellular localization of FKHRL1 with the histological subtype of the carcinomas, only the FKHRL1-positive cases were used for statistical analysis. Among the papillotubular carcinoma and scirrhous carcinoma cases, approximately 80% of the cases in each subtype showed nuclear-targeted (*i.e.*, activated) FKHRL1, whereas less than half of the cases of solid-tubular carcinomas had nuclear FKHRL1 (37%), $P < 0.001$; < 0.001, respectively (Fig. 2B: a-f and Table 3).

To confirm whether the FKHRL1-localization correlated to the activation status of Akt in breast carcinoma, 10 cases of invasive ductal carcinoma with typical FKHRL1 expression were chosen for P-Akt staining. In 5 cases which expressed nuclear FKHRL1, phosphorylated Akt was not detected on the cancer cells, as expected, whereas it was expressed and colocalized on the cells in the most cases expressing cytoplasmic FKHRL1 (among 5 cases, only one case negative for P-Akt, $P < 0.05$), see Fig. 2 C, a-d). Thus, phospho-Akt was considered to be a major kinase in the phosphorylation of FKHRL1, and, therefore, it was also reconfirmed that the intracellular localization of FKHRL1 directly reflects its activation status inside neoplastic cells.

Activation of FKHRL1 correlated with lymph nodal metastasis of breast cancer. To investigate the biological significance of nuclear-targeted (activated) FKHRL1 in breast cancers, the relationship between the activation status of FKHRL1 and several clinicopathological parameters, including the tumor size, clinical stages, lymph nodal metastasis, age of patient, depth of invasion, vascular invasion, and mitotic index were statistically evaluated. As shown in Table 1, 93 cases out of a total of 112 cases of invasive ductal carcinomas were positive for FKHRL1, but only 79 of the 93 cases were available for statistical analyses, because there was a lack of clinical information on the remaining 14 cases. The frequency of lymph nodal metastasis showed a distinct correlation to the activation status of FKHRL1 among the clinicopathological parameters mentioned above. As shown in Table 4, FKHRL1 was activated (represented by a nuclear-imported signal) on cancer cells in 33 out of 35 cases (94%) with lymph node metastasis, while no obvious difference in the activation of FKHRL1 was observed in the cases without nodal metastasis (among 44 cases, 23 cases had the active form and 21 cases had the inactive form, as shown in Table 4). The frequency of lymph nodal metastasis was higher in the cases which expressed activated FKHRL1 than in the cases with inactivated FKHRL1 ($P < 0.0001$; Table 4).

In addition to lymph nodal metastasis, the size of the primary tumor showed weak correlation with the activation status of FKHRL1 ($P = 0.035$, < 0.05), but it was not confirmable, because the numbers of cases were insufficient. A non-parametric analysis focusing on the tumor size (classified into T1 to T4 following the clinical TNM classification: UICC, Fifth Edition, 1997; T1; 26 cases, T2; 33 cases, T3; 8 cases, T4; 9 cases) and activation status of FKHRL1 (nuclear-positive or negative), revealed that distribution of the "T" stages (T1-T4) seemed to be different between the nuclear-positive and

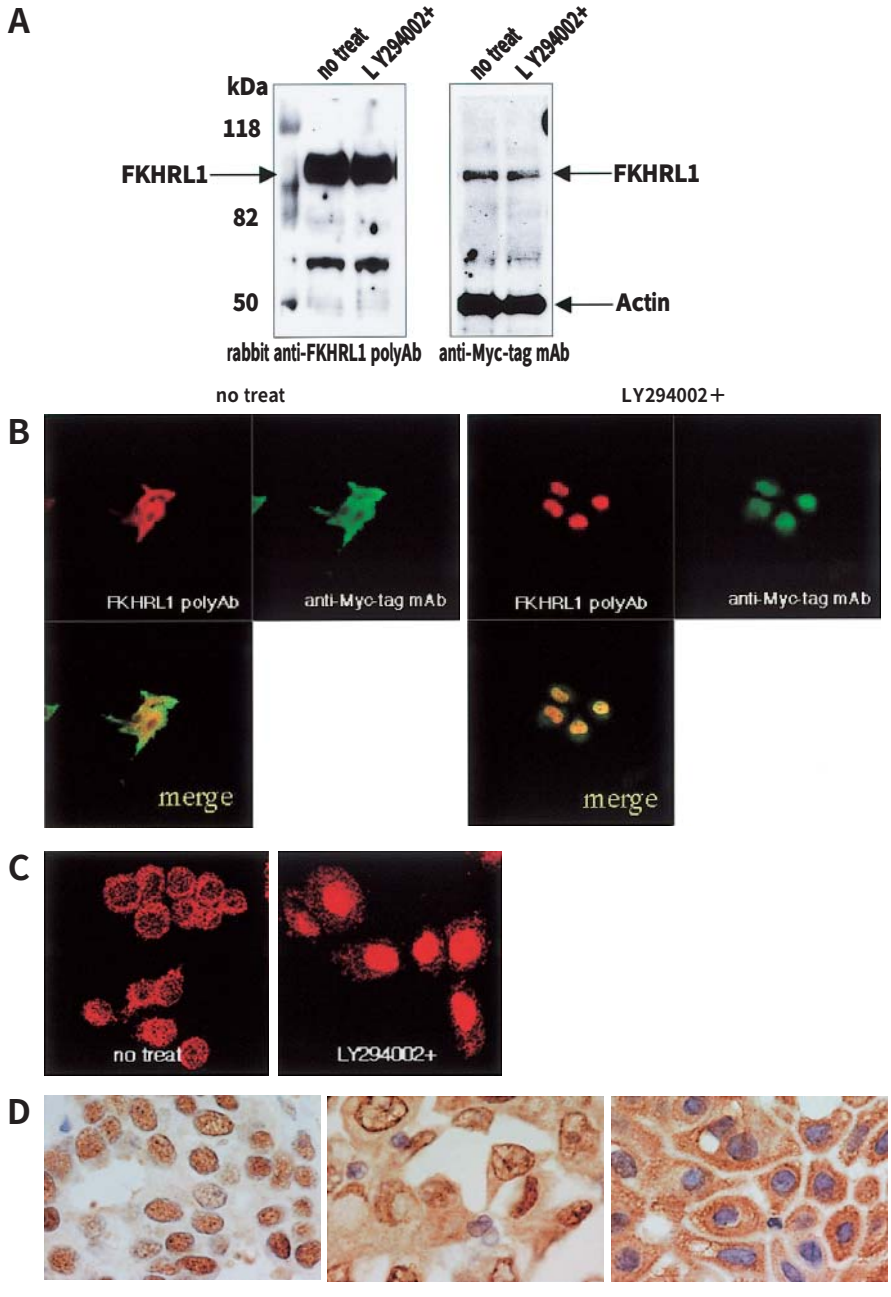

Fig. 1 Specificity of rabbit anti-FKHRL1 polyclonal antibody. **A**, Cellular lysates from BHK cells transfected with pcDNA3-myc FKHRL1 were analyzed by Western blotting by probing with the novel anti-FKHRL1 antibody (left). The same filter was probed with the anti-Myc-epitope-tagged antibody (9E10) (right panel). The bands comigrating in the same molecular weight, which corresponded to the FKHRL1 protein, were detected by both antibodies (~100-kDa). **B**, Double immunofluorescence using both the novel anti-FKHRL1 antibody and 9E10 on the BHK cells transfected with pcDNA3-myc FKHRL1 with or without Ly294002. The signal detected by the novel anti-FKHRL1 antibody was labeled with Cy3 (red), and the signal detected by 9E10 was labeled with FITC (green). The signals detected by each antibody were precisely colocalized to each other in their native condition (cytoplasmic localization; left panel) or in the presence of Ly294002 (nuclear localization; right panel). **C**, Detection of endogenous FKHRL1 in the human breast carcinoma cell line, T47D. The nuclear translocation of endogenous FKHRL1 was successfully detected on T47D cells with the novel antibody when they were treated with LY294002. **D**, The differential intracellular localization of endogenous FKHRL1 on breast cancer cells in paraffin embedded specimens detected by the novel anti-FKHRL1 antibody. Nuclear signal alone (left), predominantly nuclear (middle), cytoplasmic (right) localization of FKHRL1 was observed. The former 2 patterns were regarded as nuclear-positive FKHRL1, whereas the cytoplasmic pattern was regarded as nuclear-negative FKHRL1.

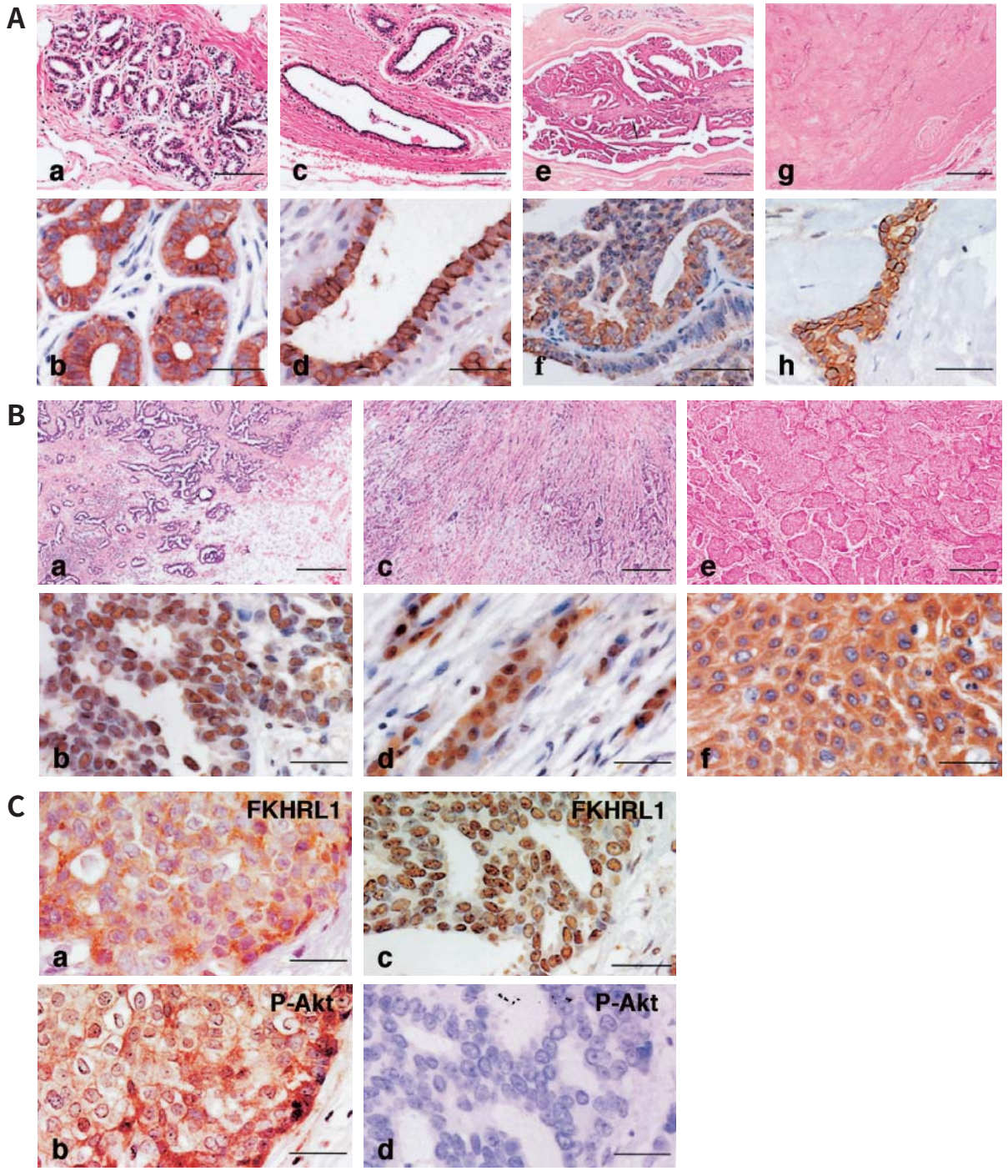

Fig. 2 A, Immunohistochemical analysis of FKHRL1 expression. Cytoplasmic staining in normal lobular (b, bar indicates 60 μm), normal ductal (d, bar indicates 60 μm) epithelium of the breast and cytoplasmic staining in intraductal papilloma (f, bar indicates 60 μm), fibroadenoma (h, bar indicates 60 μm) of the breast was shown. For HE, see a, c (bars indicate 100 μm) and e, g (bars indicate 500 μm), respectively. B, Nuclear staining of FKHRL1 in papillotubular (b, bar indicates 30 μm), scirrhous carcinoma (d, bar indicates 30 μm) and cytoplasmic staining in solid-tubular carcinoma (f, bar indicates 30 μm) of the breast. For HE, see a, c, and e (bars indicate 400 μm), respectively. C, P-Akt expressed in the specimen with cytoplasmic expression of FKHRL1 (a and b bars indicate 30 μm), but absent in the specimen with nuclear-targeted FKHRL1 expression (c and d bars indicate 30 μm).

Table 1 The expression of FKHRL1 in benign and malignant breast tumors

	No. of total cases	No. of positive cases
Intraductal papilloma	17	17 (100%)
Fibroadenoma	34	34 (100%)
Invasive ductal carcinoma	112	93 (83%)

nuclear-negative cases. Tumors in the nuclear-positive cases tended to be larger in size than those in the negative cases (frequency of the nuclear-positive cases per total FKHRL1-positive cases; 54% in T1 stage, 79% in T2, 75% in T3, and 100% in T4, data not shown). The remaining clinicopathological parameters did not show significant correlation, and the relationship between

Table 2 The intracellular localization of FKHRL1 in benign and malignant tumors of the breast

	No. of positive cases	Intracellular localization	
		Nuclear-positive (active)	Nuclear-negative (inactive)
Intraductal papilloma	17	1 (6%)	16 (94%)
Fibroadenoma	34	3 (9%) *	31 (91%) **
Invasive ductal carcinoma	93	66 (71%) *	27 (29%) **

*$P < 0.0001$; **$P < 0.0001$.

Table 3 The intracellular localization of FKHRL1 in invasive ductal carcinoma of the breast

	No. of positive cases	Intracellular localization	
		Nuclear-positive (active)	Nuclear-negative (inactive)
Papillotubular	38	30 (79%)	8 (21%)
Scirrhous	36	29 (81%) *	7 (19%) **
Solid-tubular	19	7 (37%) *	12 (63%) **

*$P < 0.0001$; **$P < 0.0001$.

Table 4 Relationship between lymph node metastasis and intracellular localization of FKHRL1 in FKHRL1-positive breast carcinomas

	No. of FKHRL1 positive cases	Intracellular localization	
		Nuclear-positive (active)	Nuclear-negative (inactive)
Lypho node metastasis			
+	35	33 (94%) *	2 (6%)
−	44	23 (52%) *	21 (48%)

*$P < 0.0001$.

FKHRL1 and the prognosis of the patients could not be accurately evaluated because the observation period was too short.

Discussion

Proto-oncogene Akt has been reported to be the key molecule which contributes to the development or neoplastic changes of tumors [22, 23]. These previous studies demonstrated that the activation of Akt inhibited the apoptosis of mammary epithelial cells and prolonged cell survival, which is considered to be important for the growth of breast tumors [24–26]. The constitutive activation of Akt alone interfered with normal mammary gland involution, however, it seemed to be insufficient to initiate tumors [26]. Moreover, the coexpression of constitutively activated Akt with the mutative polyomavirus middle T antigen resulted in the acceleration of mammary tumor growth, but did not provide the metastatic feature of tumors [26]. Thus, the oncogenesis of breast tumors is quite complicated, and cannot be simply explained by single gene alteration. Functional analyses of the Akt identified forkhead family genes, including FKHRL1, as a specific target of Akt [9–10, 27–29]. The phosphorylation of FKHRL1 induced by Akt inactivates FKHRL1 and results in its nuclear exclusion, sequestering it at the cytoplasm [9, 10]. The inactivation of FKHRL1 by this mechanism is generally explained to cause a decrease of apoptosis via the FasL-mediated death signal, and inhibits the cell cycle arrest by preventing the induction of p27 kip1 or p130 [15, 17]. However, the phosphorylated form of FKHRL1 was recognized both in cell survival and apoptosis [16, 12]. Inhibition of FKHRL1 activity by Akt-mediated phosphorylation is possibly one of the critical events by which survival factors prevent hemotopoietic cells from initiating their intrinsic apoptotic program [16]. While another report showed that phosphorylation of FKHRL1 threonine-32 is responsible for direct ROCK activation in inducing apoptosis of T47D cells [12]. The biological function of FKHRL1 seems to be still controversial, and information about the *in vivo* expression of FKHRL1 is considered to be necessary for understanding the oncogenesis of human mammary gland neoplasia. We therefore investigated the endogenous expression of FKHRL1 in benign and malignant mammary gland epithelial tumors to reveal the activation status of FKHRL1 on neoplastic cells by generating the novel paraffin-section-available antibody. Our results can be summarized as follows. First, FKHRL1 was broadly expressed in all of the benign epithelial tumors which were examined, including intraductal papillomas and fibroadenomas. In these benign tumors, FKHRL1 was detected as an inactivated form in most of the cells (*i.e.*, the nuclear-negative, cytoplasmic pattern) with the similar pattern to non-neoplastic mammary gland and ducts. Second, FKHRL1 was also expressed in over 80% of breast carcinomas; however, cancer cells predominantly expressed the activated form of FKHRL1 (nuclear-positive pattern), unlike in benign tumors. Although the number of cases we examined in this study still seems to be insufficient, this specific phenomenon was quite remarkable, especially in the scirrhous and papillotubular subtypes, and, therefore, the activation of FKHRL1 might be one of the unique characteristics in these 2 subtypes of invasive carcinomas. Finally, the cases of carcinomas with lymph nodal metastasis were mainly comprised of cases expressing activated FKHRL1 (over 90%); the cancer cells with activated FKHRL1 tended to metastasize to lymph nodes with high frequency. It is difficult to provide a concrete mechanism to explain this phenomenon in the present study, however, this finding may suggest that FKHRL1 could be kept active or could be reactivated in cancer cells by an unknown trigger. As a speculation, activated FKHRL1 may be involved in recruiting metastasis-related molecules instead of inducing apoptotic genes, such as FasL and Bim. In fact, in our study, the apoptosis markers such as cleaved caspase-3, cleaved caspase-9 and FasL, were not distinctly detected on the cancer cells with activated FKHRL1 (data not shown). Moreover, there was no distinct inverse correlation between the Ki-67 expression and nuclear FKHRL1 expression on these cancer cells by double immunofluorescence using both anti-FKHRL1 and MIB-1 (unpublished data, Kondo *et al.*).

Although the total aspect of FKHRL1, including the downstream target of activated FKHRL1 in these cells still remains unclear, activation of FKHRL1 in invasive ductal carcinoma of the breast, along with the finding that cases with nuclear-targeted FKHRL1 tend to metastasize to lymph nodes with high frequency, suggest that activated FKHRL1 may play multiple functions in the progression of mammary gland tumors *in vivo*. Further studies are needed to clarify these multiple functions.

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