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Original contribution

Prognostic impact of GATA binding protein-3 expression in primary lung adenocarcinoma☆,☆☆

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Summary GATA binding protein-3 (GATA3) is a transcription factor that regulates cell differentiation and maintenance in some types of normal cells. This study aimed to investigate the association between GATA3 expression and primary lung adenocarcinoma and to clarify the clinical significance of GATA3 expression in lung adenocarcinoma. Immunohistochemical GATA3 expression was evaluated using completely resected lung adenocarcinoma samples from 95 cases. GATA3 immunohistochemical staining was performed and scored. Associations between clinicopathological factors and GATA3 expression were analyzed by using the χ^2 test and Fisher exact test. The Kaplan-Meier method was used to analyze overall survival (OS) and disease-free survival (DFS). Forty-nine cases expressed high levels of GATA3, which were associated with lymphatic invasion ($P = .003$). In univariate and multivariate analyses, vascular invasion ($P < .001$) and high GATA3 expression ($P = .023$) were identified as independent risk factors for OS. Higher pathological stages ($P = .012$), vascular invasion ($P = .010$), and high GATA3 expression ($P = .009$) were identified as independent risk factors for DFS. The high GATA3 expression group exhibited statistically worse OS ($P = .031$) and DFS ($P = .011$) than the low-expression group based on the Kaplan-Meier curves. In resected lung adenocarcinoma, high GATA3 expression is associated with poorer prognosis for both OS and DFS. Therefore, the immunohistochemical evaluation of GATA3 represents a potentially useful prognostic tool for postoperative patients.

1. Introduction

The number of newly diagnosed lung cancer patients was 1.8 million in 2012 worldwide, with this cancer representing 13% of all cancers. In addition, lung cancer is a major leading cause of cancer-associated death worldwide [1]. In recent years, heterogeneity of adenocarcinoma has been in the spotlight, with predominant subtypes among adenocarcinomas

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being emphasized in relation to the prognosis of patients or selection of molecular target medicine [2-4].

GATA binding protein-3 (GATA3) is a member of the GATA family, which is composed of GATA1 to GATA6. GATA3 is a transcription factor that is made up of 443 or 444 amino acids and has two transactivation domains and two Zinc finger (Zf) motifs. The distal Zf motif binds to the canonical GATA motif [(A/T) GATA (A/G)]. GATA3 regulates target genes, triggering specific cell proliferation and differentiation [5,6]. GATA3 expression has been reported in many tissues and cell types, especially mammary luminal gland, T helper 2 (Th2) lymphocytes, central nervous system, kidney, and hair follicles of the skin [7,8]. In particular, GATA3 plays decisive roles in the differentiation and maintenance of normal luminal cells and the development of Th2 lymphocyte [5,9].

Several recent studies demonstrated that GATA3 expression is associated with neoplasms. For example, in breast and bladder cancer, decreased GATA3 expressions are associated with poor prognosis [10,11]. On the other hand, pancreatic cancer cells present high GATA3 mRNA and protein expression levels compared to non-neoplastic cells [12], and neuroblastoma patients with high GATA3 expression present with a poorer survival [13]. In a similar fashion, the authors demonstrated high GATA3 expression associated with poorer survival in soft tissue sarcomas [14]. However, reports are not available on GATA3 expression in relation to clinicopathological features and patient survival rates in primary lung cancer. Considering biological characteristics, this study was designed to investigate the association between GATA3 expression and lung adenocarcinoma. The results are expected to demonstrate the potential use of immunohistochemical GATA3 expression as a prognostic tool for patients with lung adenocarcinoma.

2. Materials and methods

2.1. Patients and tissue samples

This study included 95 lung adenocarcinoma samples, which were resected at Kurume University Hospital (Kurume, Japan) from January 2007 to December 2009. The following cases were excluded from this study beforehand: (1) cases who had undergone chemo- and/or radiotherapy before surgery, (2) cases who underwent incomplete resection, (3) cases at pathological stages IIIB and IV, and (4) cases with metachronous lung cancer. Tumor staging was performed according to the 7th edition of the TNM classification of the Union for International Cancer Control [15]. Histological predominant subtypes of adenocarcinoma were reclassified for each case according to the 2015 WHO 4th edition classification [16]. In addition, cases were divided into 3 groups, including low grade, intermediate grade, and high grade according to each subtype, following the previously described divisions [2]. Lymphatic/vascular invasion was evaluated by using immunohistochemistry (IHC) with D2-40 monoclonal antibody (mAb) (Nichirei, Tokyo, Japan) and Elastica van Gieson staining. The slides were evaluated by 2 pathologists (H.M. and K.O.) who were blinded to the patient outcomes. This study received the approval by the Research Ethics Committee of Kurume University, which conforms to the guidelines of the Declaration of Helsinki.

2.2. Cell culture

Human lung cancer cell lines A549 (adenocarcinoma), PC-9 (adenocarcinoma), and QG56 (squamous cell carcinoma) were cultured in D-MEM (Wako, Japan) and 11–18 (adenocarcinoma) was cultured in RPMI-1640 (Wako) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution. All cell lines were cultured in a 5% CO_2 incubator at 37°C.

2.3. Reverse-transcription polymerase chain reaction

Total RNA (1.0 μg) was extracted from each cell line using TRIzole Reagent (Invitrogen, Carlsbad, USA). This extracted RNA was reverse-transcribed, and cDNA was synthesized using Superscript VILO cDNA Synthesis Kit (Invitrogen). RT-PCR was performed by using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, USA) on TaKaRa Thermal Cycler Dice Touch (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were described as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, then 72°C for 10 minutes. The primers are listed in the Supplementary Table.

2.4. Western blotting

All cell lines were lysed in M-PER (Thermo Fisher Scientific, Waltham, USA) to obtain whole protein, or in NE-PER (Thermo Fisher Scientific) to extract nuclear and cytoplasmic proteins separately. Each protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blotted overnight at 4°C with the following antibodies: GATA3 XP mAb (1:1000; D13C9, Cell Signaling Technology [CST], Tokyo, Japan), β-actin mAb (1:1000; 13E5, CST) and CREB mAb (1:1000; 48H2, CST). Anti-rabbit IgG, HRP-linked Antibody (1:2000, CST) was used for secondary antibody. The signals ware visualized with Western Lightning ECL Pro (PerkinElmer, Waltham, USA).

2.5. IHC and immunocytochemistry

Formalin-fixed, paraffin-embedded tissue samples were sectioned at a thickness of 2.5 μm, which were deparaffinized and dehydrated. For antigen retrieval, the slides were

Table 1 Patient characteristics

Characteristic	All (n = 95)	%
Sex		
Male	40	42.1%
Female	55	57.9%
Age (y)		
Median [range]	66 [38–87]	
<66	43	45.3%
≥66	52	54.7%
Smoking status		
Never smoker	51	53.7%
Past/current smoker	44	46.3%
Serum CEA (ng/mL) (n = 92)		
Median [range]	3.2 [0.5–87.5]	
<5.0	61	66.3%
≥5.0	31	33.7%
Tumor diameter (cm)		
Median [range]	2.0 [0.7–8.4]	
<2.0	38	40.0%
≥2.0	57	60.0%
Pathological stage		
IA	51	53.7%
IB	18	18.9%
IIA	10	10.5%
IIB	5	5.3%
IIIA	11	11.6%
Lymph node status		
N0	83	87.4%
N1	3	3.2%
N2	9	9.5%
Surgical procedure		
Wedge resection	3	3.2%
Segmentectomy	3	3.2%
Lobectomy	83	87.4%
Bilobectomy	3	3.2%
Pneumonectomy	3	3.2%
Pleural invasion		
pl 0	70	73.7%
pl 1	17	17.9%
pl 2	5	5.3%
pl 3	3	3.2%
Lymphatic invasion		
ly −	67	70.5%
ly +	28	29.5%
Vascular invasion		
v −	62	65.3%
v +	33	34.7%
Predominant subtypes		
Adenocarcinoma in situ	3	3.2%
Lepidic predominant	10	10.5%
Papillary predominant	48	50.5%
Acinar predominant	20	21.1%
Solid predominant	7	7.4%
Micropapillary predominant	2	2.1%
Invasive mucinous adenocarcinoma	5	5.3%
Adjuvant therapy		
−	75	78.9%
+	20	21.1%
Recurrence		
−	61	64.2%
+	34	35.8%

Abbreviations: CEA, carcinoembryonic antigen.

pretreated in a microwave oven at 95°C for 40 minutes in Target Retrieval Solution buffer, pH 9.0 (Dako, Tokyo, Japan). Then, 3% H_2O_2 was used for 5 minutes to block endogenous peroxidase activity. The slides were incubated with primary antibody, GATA3 XP rabbit mAb (1:100; D13C9, CST) in a humidified chamber at 4°C overnight, and with secondary antibody, REAL EnVision Detection Systems (Dako) for 30 minutes. The signals were visualized using 3,3′-diaminobenzidine chromogen (Dako). The same protocol was applied to immunocytochemistry. As negative controls, we performed IHC with normal rabbit immunoglobulin (IgG). Small Th2 lymphocytes around the tumor were used as internal positive controls.

2.6. Analysis of immunohistochemical GATA3 positive rates and definition of the cut-off value

We defined immunohistochemically GATA3-positive cells as tumor cells whose nuclei were at least weakly stained, as previously described [14]. GATA3 expression was evaluated as the highest percentage of positively stained cells among all tumor cells in a high-power field (×400) where the pathologists selected. The cut-off value was set at 6.8%, which was the median of GATA3 positive rates (Fig. 2). Cases that exceeded the 6.8% threshold were designated as the high GATA3 expression group.

2.7. Statistical analysis

Associations between GATA3 expression and clinicopathological variables were evaluated using χ^2 test or Fisher exact test. The grades of adenocarcinoma were divided into low and intermediate versus high grade [2]. The overall survival (OS) was measured from the date of surgery to the date of death by any cause or last follow-up. Disease-free survival (DFS) was measured from the date of surgery to the date when recurrence was confirmed by image diagnosis (such as computed tomography and positron-emission tomography) or pathological diagnosis, when possible. The Kaplan-Meier method was used to calculate the survival rate of patients. Differences between 2 groups were evaluated by the log-rank test. The Cox proportional hazards model was used to detect prognostic factors by univariate and multivariate analyses. All statistical analyses were carried out using JMP version 11 (SAS Institute, Tokyo, Japan). P values less than .05 were considered statistically significant.

3. Results

3.1. Patient characteristics

The clinicopathological characteristics of 95 patients are summarized in Table 1. The median postoperative follow-up period was 63 months (range, 2-90). This study included 40 males (42.1%) and 55 females (57.9%), with a mean age of 66 years (range, 38-87). There were 69 (72.6%) stage I cases and 26 (27.4%) stage II and III cases. The numbers of the cases with lymphatic and vascular invasion were 28 (29.5%) and 30 (31.6%), respectively. Eighty-one cases (85.3%) were low/intermediate grade. Overall, 34 patients (35.7%) experienced recurrence during the follow-up period.

3.2. GATA3 expression in lung adenocarcinoma cell lines

RT-PCR analysis revealed that lung cancer cell lines, including PC-9 and QG56 expressed GATA3 mRNA, but A549 and 11–18 cell lines had no GATA3 mRNA expression (Fig. 1A). Western blotting (WB) using whole-cell lysates demonstrated single bands specific to GATA3 protein (Fig. 1B) in PC-9 and QG56 cell lines. And WB using separately extracted proteins from nucleus and cytoplasm revealed localization of GATA3 was mainly not cytoplasm but nucleus (Fig. 1C). Moreover, immunocytochemical analysis using these cell lines, PC-9 and QG56 showed GATA3 expression in nucleus and not in cytoplasm. The results of immunocytochemical analysis were identical with that of WB (Fig. 1D).

3.3. Analysis of immunohistochemical GATA3 expression in lung adenocarcinoma

GATA3 positive rates in each case ranged from 0% to 95% (Fig. 2). Seventy cases (73.7%) presented immunohistochemically positive cells in at least one field and were able to be evaluated. Representative IHC microphotographs of GATA3 staining are shown in Fig. 3. Based on the 6.8% threshold, 49 cases (51.6%) were categorized in the high GATA3 expression group.

3.4. Associations between GATA3 expression and clinicopathological characteristics

Associations between GATA3 expression and clinicopathological characteristics are summarized in Table 2. Only lymphatic invasion (P = .003) was more frequently identified in the high GATA3 expression group. Yet there was no association between GATA3 expression and lymph node metastasis

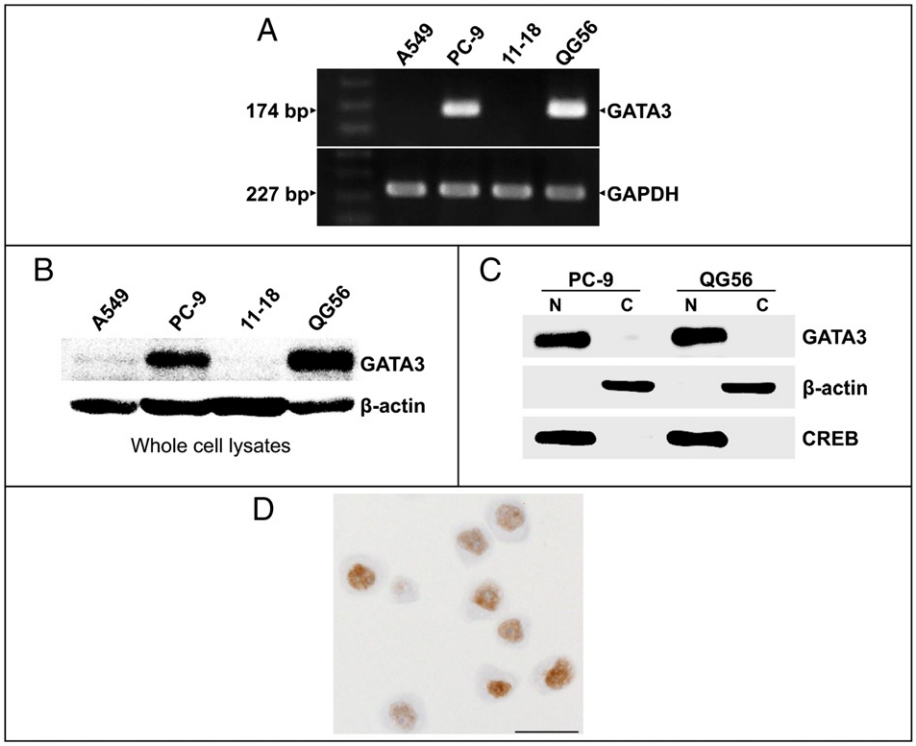

Fig. 1 A, GATA3 mRNA expressions are recognized in PC-9 (adenocarcinoma) and QG56 (squamous cell carcinoma) lung cancer cell lines. B and C, Western blotting analyses. PC-9 (adenocarcinoma) and QG56 (squamous cell carcinoma) represent single bands specific to GATA3 protein extracted from whole cell lysates (B) and nucleus (C). N: nuclear protein, Cy: cytoplasmic protein. D, Immunocytochemistry of GATA3 with PC-9 lung adenocarcinoma cell line. Nuclei of PC-9 cells are positive for GATA3. (Bar indicates 20 μm.)

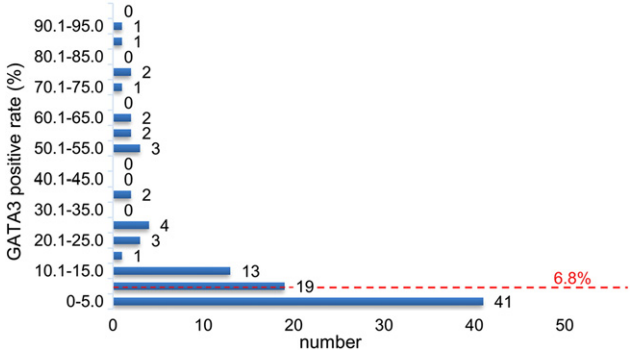

Fig. 2 Distribution of GATA binding protein (GATA3) positive rates and definition of GATA3 cut-off value, defined as 6.8%, which is the median positive rate of all cases.

(P = .907). Other variables listed in Table 2, including the grade of adenocarcinoma (P = .252), were not statistically associated with GATA3 expression.

3.5. Survival curves and univariate and multivariate analysis in each GATA3 expression group

The Kaplan–Meier curves demonstrate that OS was significantly shorter for patients presenting with lung adenocarcinoma with high GATA3 expression (P = .031) when compared with patients with low GATA3 expression (Fig. 4). Patients in the high GATA3 expression group also exhibited significantly shorter DFS (P = .011). Univariate analysis for OS and DFS revealed that high GATA3 expression (P = .029 and .011, respectively) was a poor prognostic factor (Table 3, upper). Multivariate analysis demonstrated that vascular invasion (P < .001) and high GATA3 expression (P = .023) were independent poor prognostic factors for OS. Higher pathological stage (P = .012), vascular invasion (P = .010), and high GATA3 expression (P = .009) were poor prognostic factors for DFS (Table 3, lower).

4. Discussion

This study demonstrated that lung adenocarcinoma cell line PC-9 had GATA3 mRNA and protein expression in nucleus. And lung adenocarcinoma cases present varying degrees of immunohistochemical GATA3 expression. Moreover, high GATA3 expression is associated with lymphatic invasion, and GATA3 expression isn't influenced by the grade of adenocarcinoma. Kaplan–Meier curves demonstrated that patients with high GATA3 expression had shorter OS and DFS. In addition, univariate and multivariate analyses indicated that high GATA3 expression is a poor independent prognostic factor for lung adenocarcinoma.

Some lung adenocarcinoma cases were immunohistochemically positive for GATA3 in this study. Miettinen et al evaluated GATA3 expression in many neoplasms, which arose from many organs [8]. The authors found that 6 out of 71 lung adenocarcinoma cases were positive for GATA3. However, the positive rates in their report varied widely. In this study, GATA3 positive rates were consistently evaluated as the highest percentage of the positive cells in high-power fields.

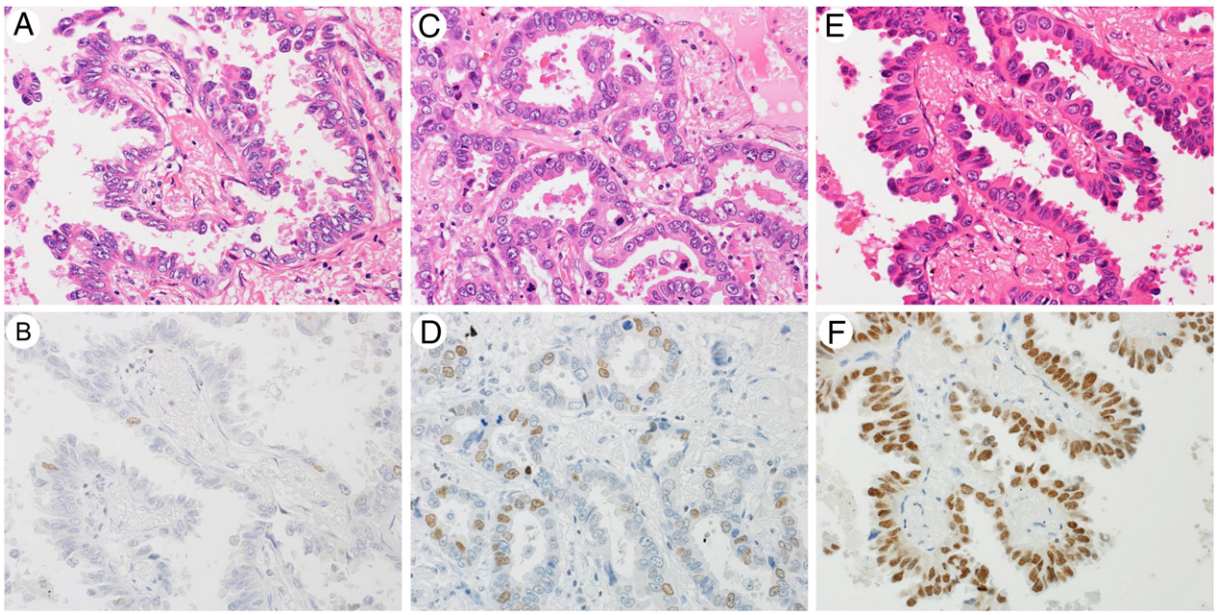

Fig. 3 Hematoxylin and eosin staining (original magnification ×400) (upper row) and GATA3 immunohistochemistry (IHC) (original magnification ×400) (lower row). A and B, Papillary component; microphotographs show that few nuclear positive cells are recognized in papillary adenocarcinoma component. C and D, Acinar component; positive rate for this case is 25% in acinar adenocarcinoma component. E and F, Papillary component; almost all neoplastic cells are immunohistochemically positive in papillary adenocarcinoma component.

Table 2 Patient characteristics and association with GATA3 expression

Characteristic	GATA3 high $n = 49$ (51.6%)	GATA3 low $n = 46$ (48.4%)	P (χ^2)
Sex			.569
Male	22	18	
Female	27	28	
Age (y)			.453
<66	24	19	
≥66	25	27	
Smoking status			.900
Never smoker	26	25	
Past/Current smoker	23	21	
Serum CEA (ng/mL) (n = 92)			.608
<5.0	30	31	
≥5.0	17	14	
Tumor diameter (cm)			.557
<2.0	21	17	
≥2.0	28	29	
Lymph node status			.907
N 0	43	40	
N +	6	6	
Pathological stage			.464
Stage I	34	35	
Stage II and III	15	11	
Grading of adenocarcinoma			.252 [a]
Low/Intermediate grade	44	37	
High grade	5	9	
Pleural invasion			.069
pl −	40	30	
pl +	9	16	
Lymphatic invasion			.003 *
ly −	28	39	
ly +	21	7	
Vascular invasion			.660
v −	33	29	
v +	16	17	

NOTE. Low/intermediate grade include adenocarcinoma in situ, lepidic, papillary and acinar pattern; high grade includes solid, micropapillary, invasive mucinous pattern.

* $P < .05$.

[a] Fisher's exact test.

Only a few samples had diffuse positive cells in all fields, but most samples had heterogeneity of positive cells in an identical sample. With respect to the heterogeneity, we are confident that our GATA3 positive rate was representative and easy to evaluate.

In this study, GATA3-positive cells were defined as tumor cells whose nuclei were positive, but not cytoplasm, whereas Gulbinas et al reported cytoplasmic GATA3 expression in pancreatic cancer [12]. Indeed, the cytoplasmic GATA3 expressions were also investigated by IHC with GATA3 mAb (D13C9, CST). Twenty in all 95 cases had partial cytoplasmic expression of GATA3, which was confirmed by using another GATA3 mAb (L50–823, Biocare Medical, USA). Although there were some differences in intensity, each cytoplasmic expression basically showed a similar tendency. However, the cytoplasmic expression of GATA3 didn't show statistical significance associated with clinicopathological features including OS and DFS (Supplementary Figure). Although GATA3 expression only in nucleus is a prognostic factor, its expression in cytoplasm might be possibly significant in tumor proliferation or progression. Further research is needed to investigate the localization of GATA3 and its association to tumor biology.

In a carcinogenic view, authors speculated that up-regulated GATA3 cooperates with Smad3 and activates TGF-β in the same way as Th2 lymphocytes [17], which increases the Smad3/4 complex. This complex may up-regulate N-cadherin (NCAD) [18]. Some studies demonstrated the relationship between the TGF-β pathway and epithelial-mesenchymal transition (EMT) pathway [19,20]. In this study, we tried to show the relationship between GATA3 and NCAD as an EMT marker using IHC analysis with NCAD mAb. But the number of NCAD- positive adenocarcinomas was only 6, and we couldn't clarify the relationship (data not shown). Further study with large numbers of cases may show the relationship between GATA3 and NCAD, and explain why high GATA3 expression is a poor prognostic factor in patients with lung adenocarcinoma. On the other hand, some reports revealed the association between GATA3 and the Notch pathway [21-23]. The Notch1/2 pathway including RBPJ (Recombination signal Binding Protein for immunoglobulin kappa J region) plays an important role in the differentiation of Th2 lymphocyte. RBPJ activates GATA3 promoter, then GATA3 is induced [24]. In this study, we performed Notch-1 IHC analysis with Notch-1 mAb. The association between GATA3 and Notch-1 expressions were not clarified in the same way as NCAD expression in this study (data not shown). However, GATA3 may correlate with other Notch proteins like Notch-2,3,4 and Notch ligand, and these factors deserve further research.

To date, several studies reported that GATA3 IHC is useful for distinguishing metastatic breast and urothelial carcinoma from primary lung carcinoma [25,26]. While breast and urothelial carcinoma tend to be positive for GATA3, this study revealed that the number of cases of primary lung adenocarcinoma with GATA3 expression was not small. Therefore, GATA3 IHC, in combination with other parameters, is recommended when pathologists use IHC to distinguish metastatic carcinoma from primary lung carcinoma.

Recently, SB010, which is a GATA3-specific DNA enzyme, was reported to be effective against bronchial asthma in a phase IIb study [27]. hgd40 is the active product of SB010, which cleaves GATA3-specific mRNA and regulates GATA3 protein expression in lymphocytes. While the function of GATA3 in lung adenocarcinoma is still unclear, drugs like SB010 might be effective molecular targeted therapies for patients that present with lung adenocarcinoma with high GATA3 expression.

This study presents some limitations. First, this study used retrospective data. A prospective trial is needed to confirm the

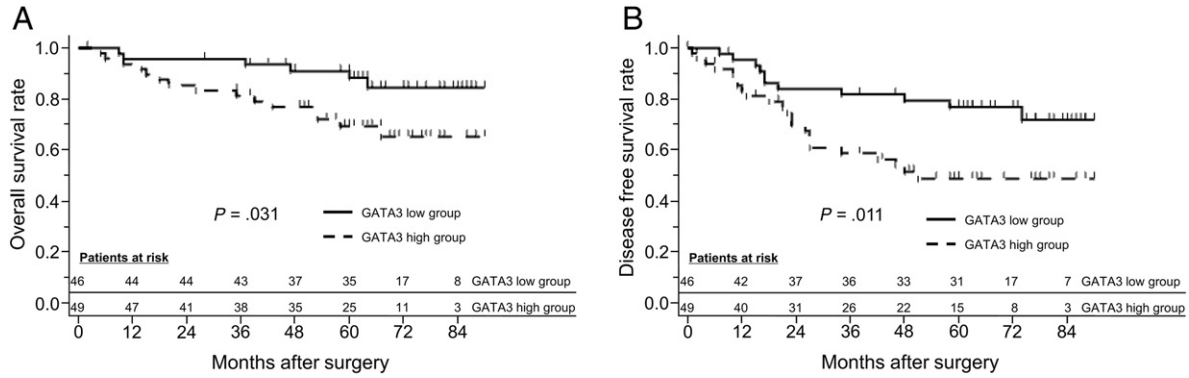

Fig. 4 Kaplan–Meier curves for OS (A) and DFS (B). Patients in the high GATA3 expression group (dotted line) presented statistically shorter OS ($P = .031$) and DFS ($P = .011$) than patients in the low GATA3 expression group (solid line).

results of this study. Secondly, the number of cases was relatively small, but the authors evaluated only adenocarcinoma cases, not including other histological subtypes. While the statistical significance of this study was sufficient, further studies with larger numbers of patients are desirable. Finally, though high GATA3 expression was significantly associated with lymphatic

Table 3 Univariate and multivariate analysis for OS and DFS

		OS		DFS	
Variables	n	HR (95% CI)	*P*	HR (95% CI)	*P*
Univariate analysis					
Sex					
Male	40	1		1	
Female	55	0.31 (0.12–0.74)	.008 *	0.71 (0.36–1.40)	.313
Smoking status					
Never smoker	51	1		1	
Past/current smoker	44	2.68 (1.12–7.08)	.027 *	1.01 (0.50–1.98)	.980
Pathological stage					
I	69	1		1	
II or III	26	3.31 (1.40–7.96)	.007 *	3.25 (1.63–6.40)	.001 *
Lymph node status					
N0	83	1		1	
N+	12	2.16 (0.71–5.51)	.163	4.25 (1.93–8.72)	< .001 *
Vascular invasion					
v −	62	1		1	
v +	33	3.36 (1.42–8.50)	.006 *	2.18 (1.10–4.30)	.026 *
Adjuvant therapy					
−	75	1		1	
+	20	1.51 (0.54–3.72)	.409	3.03 (1.47–6.01)	.003 *
GATA3 expression					
Low-expression group	46	1		1	
High-expression group	49	2.72 (1.10–7.63)	.029 *	2.47 (1.23–5.29)	.011 *
Multivariate analysis					
Pathological stage					
I	69	1		1	
II and III	26	1.98 (0.81–4.95)	.137	2.65 (1.25–5.56)	.012 *
Vascular invasion					
v −	62	1		1	
v +	33	4.76 (1.92–12.48)	< .001 *	2.56 (1.26–5.19)	.010 *
GATA3 expression					
Low-expression group	46	1		1	
High-expression group	49	2.87 (1.15–8.14)	.023 *	2.62 (1.26–5.75)	.009 *

Abbreviations: HR, hazard ratio; CI, confidence interval.
* $P < .05$.

invasion and clinical course, the specific mechanisms are not clarified. Additional functional analyses are necessary to confirm the association of high GATA3 expression with lymphatic invasion and poor prognosis.

In conclusion, the evaluation of immunohistochemical GATA3 expression could help estimate the prognosis of postoperative patients, even though high GATA3 expression is a poor independent prognostic factor for lung adenocarcinoma. In other words, the prognosis of patients with low GATA3-expressing adenocarcinoma may be good. Thus, we could easily divide high- and low-expression groups by IHC evaluation, which is a low-cost procedure that does not require complicated techniques, as a simple clinical prognostic tool for patients with lung adenocarcinoma.

Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2017.02.024.

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