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The Prognostic Significance of *Notch1* and Fatty Acid Binding Protein 7 (*FABP7*) Expression in Resected Tracheobronchial Adenoid Cystic Carcinoma: A Multicenter Retrospective Study

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Purpose

Adenoid cystic carcinoma (ACC) of the trachea and bronchus is a rare tumor. Although *MYB-NFIB* oncogene fusion and *Notch1* mutation have been identified in ACC, little is known about the expression and clinical significance of *Notch1* and its target gene fatty acid binding protein 7 (*FABP7*) in tracheobronchial ACC.

Materials and Methods

Primary tracheobronchial ACC that were resected between 1998 and 2014 were identified through the pathology and oncology database from five thoracic oncology centers in China. A tissue array was constructed from the patients' samples and the expressions of *Notch1* and *FABP7* were evaluated by immunohistochemistry. The association between the expression of both markers and survival was determined.

Results

Overexpression of *Notch1* and *FABP7*, detected in 37.8% and 38.3% of 368 patients with tracheobronchial ACC, respectively, was an independent prognostic indicator for recurrence-free survival (RFS) by multivariable Cox proportional hazard model (p=0.032 and p=0.048, respectively). Overexpression of *Notch1*, but not of *FABP7*, predicted overall survival (OS) (p=0.018). When categorized into four groups according to coexpression of *Notch1* and *FABP7*, patients with overexpression of both *Notch1* and *FABP7* belonged to the group with the shortest RFS and OS (p=0.01 and p=0.048, respectively).

Conclusion

Expression of *Notch1* and *FABP7*, and coexpression of *Notch1* and *FABP7*, is strongly associated with poor survival in resected tracheobronchial ACC. These data are consistent with the hypothesis that poor differentiation of tracheobronchial ACC correlates with the activation of Notch signaling.

Key words
Adenoid cystic carcinoma, Prognosis, Bronchi, Trachea, *Notch1*, *FABP7*

Introduction

Adenoid cystic carcinoma (ACC) of the trachea and bronchus is a rare type of cancer [1]. This histology exhibits perineural invasion and the rate of local recurrence and late metastasis is relatively high. Tubular, cribriform, and solid subtypes were three main histological growth patterns [2]. Surgical resection is the main treatment for patients with ACC. The 5-year survival rate of patients with resected tracheobronchial ACC were 52% [3,4]. Complete resection is achieved in approximately 50% of patients due to mucosal invasion [5]. Radiotherapy is recommended for patients with positive resection margin [6]. Tracheobronchial ACC is rather prone to recur locally or distantly after the surgical resection [7]. However, the prognostic factors of tracheobronchial ACC remain unknown.

A diagnostic feature of ACC is a t(6;9) rearrangement that translocates *MYB* to the *NFIB* locus [8-12]. Most ACCs overexpress *MYB*, only 30% harbor fusion transcript [13]. ACCs harbor activating *Notch1* mutations [14]. *MYB* coordinates with *Notch* in ACC [8]. *Notch* activation by gain-of-function mutation underlies the switch to poorly differentiated histology and worse clinical outcome. Fatty acid binding protein 7 (*FABP7*) is a target gene of Notch and related to poor prognosis in ACC of the salivary glands [15,16]. We assume that ACCs with high expression of *Notch1* and *FABP7* have an aggressive phenotype and poor prognosis. In order to test this hypothesis, we investigated the expression of both *Notch1* and *FABP7* of resected tracheobronchial ACC in a large retrospective study. The aim of this study is to investigate the association between expression of *Notch1* and *FABP7* and survival.

Materials and Methods

1. Patients selection

The multicenter study of rare thoracic cancer is a cooperative multicenter group composed of five hospitals in China (The First Affiliated Hospital of Guangzhou Medical University, The First Affiliated Hospital of Tsinghua University, Sun-Yet Sen University Cancer Center, The First Affiliated Hospital, Sun-Yet Sen University, and Guangdong General Hospital). All resected tracheobronchial ACC at the participating institutions from January 1998 to July 2014 were registered. Microscopic involvement of tracheal or bronchial margin was accepted if the airway was normal on gross inspection and no further length of airway could be resected. Radiotherapy of 54 Gy was administered 6 weeks after surgical resection. Patients who died of postoperative complications during the 30 days following the surgery, had history of primary ACC at other sites or secondary tumor were excluded.

2. Clinicopathological data

We analyzed the association between clinicopathological characteristics and patterns of recurrence and overall survival. Clinicopathological variables included age, sex, location of the tumor, histologic subtype, tumor size, lymph node involvement, resection margin, and postoperative treatment.

3. Immunohistochemical assay

After dewaxing in xylene and rehydrating stepwise in ethanol, sections were subjected to heat-induced antigen retrieval. Endogenous peroxidase activity and nonspecific binding were blocked with 3% H_2O_2 and nonimmune sera, respectively. Sections were then incubated with primary antibodies overnight at 4°C. The primary antibodies were used as below: cleaved *Notch1* (Val1744) antibody from Cell Signaling (Danvers, MA) and *FABP7* antibody from Abcam (Ab32423, Cambridge, MA). The next day, primary antibody was detected by Streptavidin-Biotin kit (Maixin Biotechnology, Fuzhou, China). Immunolabeled sections were visualized with 3,3´-diaminobenzidine, counterstained with hematoxylin, dehydrated, mounted and observed by means of a DM2000 microscope. Images were analyzed with Image J software. The analysis was performed by two independent pathologists (Y.G. and X.F). Scoring of *Notch1* and *FABP7* was calculated according to the staining intensity (0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining) and the percentage of tumor cells showing staining (< 25%, 25%-50%, and > 50%). To determine the cut-off point for immunohistochemical staining of *Notch1* and *FABP7*, the cohort was divided into "training set" and "validation set." Cut-off points were determined based on the results from the training set and were then proved in validation set. H-Score > 150 was considered high expression of *Notch1* and *FABP7*. This method has been described by Hilsenbeck et al. [17] to reduce the risk of type 1 error associated with multiple testing for optimal cut-off points.

4. Fluorescent *in-situ* hybridization

MYB/*NFIB* rearrangements were detected by fluorescent *in-situ* hybridization. In nuclei containing the *MYB-NFIB* fusion, green and red signals from the *MYB* and *NFIB* genes overlap in a red/green (yellow) signal. Details are available in the supplementary data.

5. Statistical analysis

The expressions of *Notch1* and *FABP7* were categorized into either "low" or "high" scores according to the criteria described above. Survival data were collected from a systematic follow-up database. Chi-squared tests were used to evaluate the association between expression of *Notch1* and *FABP7* and clinicopathological parameters. Recurrence-free survival (RFS) was defined as the time period from the date of surgery until recurrence. The overall survival (OS) was calculated from the date of diagnosis to the date of death caused by ACC or December, 2015, the follow-up cut-off date. Patients who died of causes other than ACC and patients who still alive at the time of the follow-up cut-off were censored. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazard regression model was used for univariate and multivariate analyses of survival. Significant variables ($p < 0.1$) in univariate analysis were included in multivariate analysis. All the statistical tests were two-sided. p-value ≤ 0.05 was regarded as significant. SPSS software ver. 19.0 (SPSS Inc., Chicago, IL) was used for statistical analyses.

6. Ethical statement

Ethical approval was obtained from each participating institution through institutional review board (IRB). Written informed consent for tumor sample collection and analysis was obtained from all patients.

Results

1. Patients

Patients with tracheobronchial ACC who received surgical resection have been retrospectively reviewed in five thoracic cancer center. Four hundred eleven medical records were reviewed. Twenty-three patients were excluded due to history of primary ACC at other sites or secondary tumor and

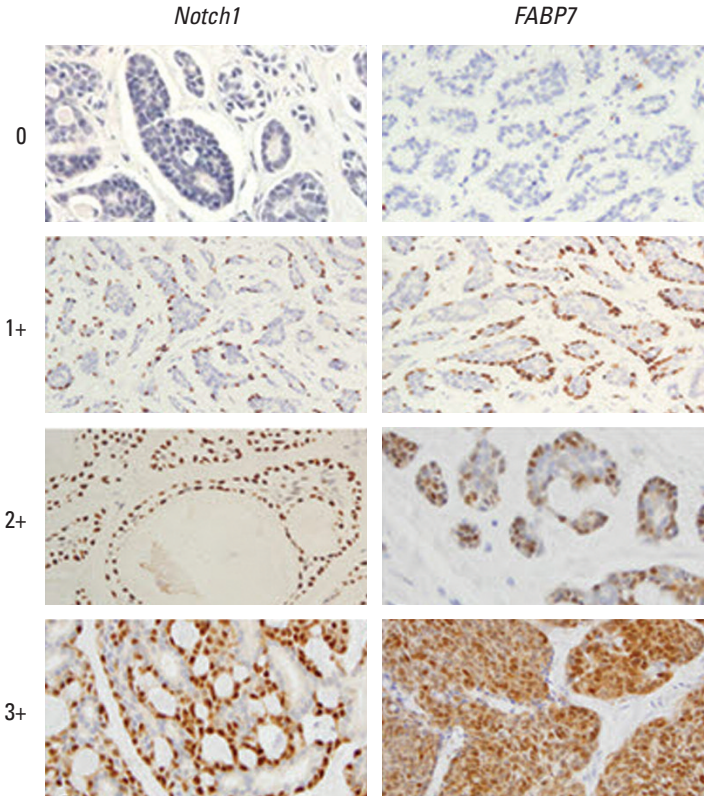

Fig. 1. Representative immunohistochemical staining intensity of *Notch1* (left column) and *FABP7* (right column) in patients with tracheobronchial adenoid cystic carcinoma. 0, no staining; 1+, mild; 2+, moderate; 3+, strong intensity of staining (×200).

Table 1. Association between *Notch1* and *FABP7* expression and clinicopathological characteristics in 368 patients with resected tracheobronchial adenoid cystic carcinoma

Characteristic	*Notch1* expression			*FABP7* expression		
	Low	High	p-value	Low	High	p-value
Age (yr)						
< 50	126	66	0.381	129	63	0.533
≥ 50	103	73		98	78	
Sex						
Male	128	75	0.863	123	80	0.367
Female	101	64		104	61	
Histologic subtype						
Cribriform/Tubular	207	87	0.006	200	94	0.012
Solid	22	52		27	47	
Margin status						
Negative	127	69	0.472	119	77	0.639
Positive	102	70		108	64	
Lymph node involvement						
Negative	122	52	0.027	114	60	0.034
Positive	33	48		35	46	
No biopsy	74	39		78	35	
Location						
Trachea	100	66	0.176	105	61	0.062
Bronchus	129	73		122	80	
Tumor size (cm)						
< 3	172	89	0.010	159	102	0.468
≥ 3	57	50		68	39	
***MYB-NFIB* gene fusion**						
Yes	70	116	0.016	65	121	0.013
No	159	23		162	20	

20 patients were excluded due to either the unavailability of an formalin fixed paraffin embedded material from our archives or the quality of the material. A total of 368 patients between January 1998 to July 2014 were included in this study. Demographics and clinical features are shown in S1 Table. The median age at presentation was 50.2 years (range, 17 to 82 years). Mean tumor size was 3.2 cm (range, 1.6 to 7.8 cm). Three hundred fifty-six patients (96.7%) were symptomatic. The mean duration of symptoms was 11.9 months. Pathological features are described in S2 Table. The types of resection for primary tracheobronchial ACC are shown in S3 Table. Sixty-one point four percent (226/368) of resected specimens had positive microscopic margins. Selective sampling lymph node metastases were found in 22.0% (81/368) of patients, most from peritracheal and subcarinal stations; nodal biopsies were not obtained in 30.7% (113/368) of resected patients. One hundred forty patients (38.0%) had no postoperative treatment. Two hundred twenty-eight patients (62.0%) received postoperative therapy. One hundred eighty-three patients (49.7%) received postoperative radiotherapy alone and 45 patients (12.3%) received the postoperative sequential radiotherapy followed by chemotherapy. *MYB-NFIB* gene fusion was identified in 186 patients (50.5%) (S4 Fig.).

2. Association of clinicopathological parameters with expression of *Notch1* and *FABP7*

Representative immunostaining of *Notch1* and *FABP7* in tracheobronchial ACC tissues are shown in Fig. 1. Activated *Notch1* were nuclear stained in cancer cells. The staining patterns of *FABP7* were most nuclear and cytoplasm in cancer cells. No expression was observed in normal bronchial epithelium. Among 368 patients who had pathological slides for central review, 139 patients (37.8%) showed *Notch1* overexpression and 141 patients (38.3%) showed *FABP7* overexpression. Associations between expression of *Notch1* and *FABP7* and clinicopathological characteristics (age, sex, tumor size, lymph node involvement, histologic subtype, tracheobronchial location, and *MYB-NFIB* gene fusion) are

Table 2. Univariate and multivariate analysis of recurrence-free survival in 368 patients with resected tracheobronchial adenoid cystic carcinoma

Variable	Univariate			Multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (yr)						
< 50	1.00		0.435			
≥ 50	1.18	0.74-1.55				
Sex						
Male	1.00		0.746			
Female	0.97	0.75-1.24				
Tracheobronchial location						
Trachea	1.00		0.722			
Bronchus	0.96	0.61-1.53				
Lymph node involvement						
Negative	1.00		0.062			
Positive	1.09	0.89-1.45				
Tumor size (cm)						
< 3	1.00		0.013	1.00		0.118
≥ 3	1.67	1.44-1.88		1.18	0.97-1.43	
Margin status						
Negative	1.00		0.003	1.00		0.007
Positive	2.35	1.91-2.65		1.90	1.63-2.21	
Histologic subtype						
Cribriform/Tubular	1.00		0.001	1.00		0.008
Solid	2.36	1.63-2.99		1.92	1.70-2.13	
Postoperative therapy						
Yes	1.00		0.003	1.00		0.010
No	2.02	1.52-2.78		1.83	1.47-2.19	
***Notch1* expression**						
Low	1.00		0.009	1.00		0.032
High	1.90	1.68-2.25		1.39	1.16-1.76	
***FABP7* expression**						
Low	1.00		0.040	1.00		0.048
High	1.37	1.11-1.63		1.34	1.11-1.62	
***Notch1*/*FABP7* expression**						
Notch1^{low}*FABP7*^{low}	1.00		0.010	1.00		0.036
Notch1^{high}*FABP7*^{low}	1.15	0.67-1.55		1.08	0.66-1.56	
Notch1^{low}*FABP7*^{high}	1.28	0.96-1.74		1.19	0.67-1.89	
Notch1^{high}*FABP7*^{high}	1.85	1.28-2.62		1.37	1.12-1.89	

HR, hazard ratio; CI, confidence interval.

shown in Table 1. *Notch1* expression was significantly associated with solid pattern (p=0.006), lymph node involvement (p=0.027) and large tumor size (p=0.01). *FABP7* expression was associated with solid pattern (p=0.012) and lymph node involvement (p=0.034). Both *Notch1* (p=0.016) and *FABP7* (p=0.013) expressions were associated with *MYB-NFIB* gene fusion.

3. Expression of *Notch1* and *FABP7* as prognostic factors in patients with tracheobronchial ACC

With a median follow-up period of 65 months (range, 18 to 156 months), the OS for all resected ACC patients was 72.0% at 5 years and 45.6% at 10 years. The 5-year and 10-year RFS was 43.2% and 12.1%.

Kaplan-Meier survival analysis was performed to evaluated the prognostic value of individual marker in the whole

Table 3. Univariate and multivariate analysis of overall survival in 368 patients with resected tracheobronchial adenoid cystic carcinoma

Variable	Univariate			Multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (yr)						
< 50	1.00		0.440			
≥ 50	1.22	0.74-1.56				
Sex						
Male	1.00		0.638			
Female	0.95	0.73-1.18				
Tracheobronchial location						
Trachea	1.00		0.431			
Bronchus	0.92	0.71-1.60				
Lymph node involvement						
Negative	1.00		0.591			
Positive	1.16	0.99-1.29				
Tumor size (cm)						
< 3	1.00		0.323			
≥ 3	1.29	0.83-1.79				
Margin status						
Negative	1.00		0.007	1.00		0.026
Positive	1.91	1.70-2.19		1.52	1.32-1.79	
Histologic subtype						
Cribriform/Tubular	1.00		0.002	1.00		0.023
Solid	2.38	1.92-2.82		1.45	1.18-1.97	
Postoperative therapy						
Yes	1.00		0.005	1.00		0.021
No	2.15	1.87-2.45		1.66	1.37-1.93	
***Notch1* expression**						
Low	1.00		0.010	1.00		0.018
High	1.67	1.38-2.12		1.59	1.27-1.90	
***FABP7* expression**						
Low	1.00		0.167	1.00		0.375
High	1.10	0.93-1.37		1.16	0.83-1.48	
***Notch1*/*FABP7* expression**						
$Notch1^{low}FABP7^{low}$	1.00		0.048	1.00		0.164
$Notch1^{high}FABP7^{low}$	1.16	0.88-1.63		1.07	0.67-1.58	
$Notch1^{low}FABP7^{high}$	1.22	0.86-1.79		1.13	0.85-1.71	
$Notch1^{high}FABP7^{high}$	1.47	1.15-1.94		1.32	1.09-2.15	

HR, hazard ratio; CI, confidence interval.

cohort. Univariable analysis for all other variable is shown in Tables 2 and 3. The results show that *Notch1* expression had significant impact on both RFS (p=0.009; hazard ratio [HR], 1.90; 95% confidence interval [CI], 1.68 to 2.25) (Fig. 2A) and OS (p=0.01; HR, 1.67; 95% CI, 1.38 to 2.12) (Fig. 3A). *FABP7* showed a trend towards poor RFS (p=0.04; HR, 1.37; 95% CI, 1.11 to 1.63) (Fig. 2B). No statistical relationship was found between OS and *FABP7* (p=0.167) (Fig. 3B). There was no significant difference of OS between tracheobronchial ACC patients with or without *MYB-NFIB* rearrangement (p=0.109) (S5 Fig.).

Variables with p-value of 0.1 or less were entered in Cox regression model for multivariable analysis. Both *Notch1* and *FABP7* were independent predictors of survival. For RFS, the five variables that remained independently substantially associated with RFS were positive resection margin (p=0.007), solid pattern (p=0.008), without postoperative therapy (p=0.010), *Notch1* overexpression (p=0.032), and

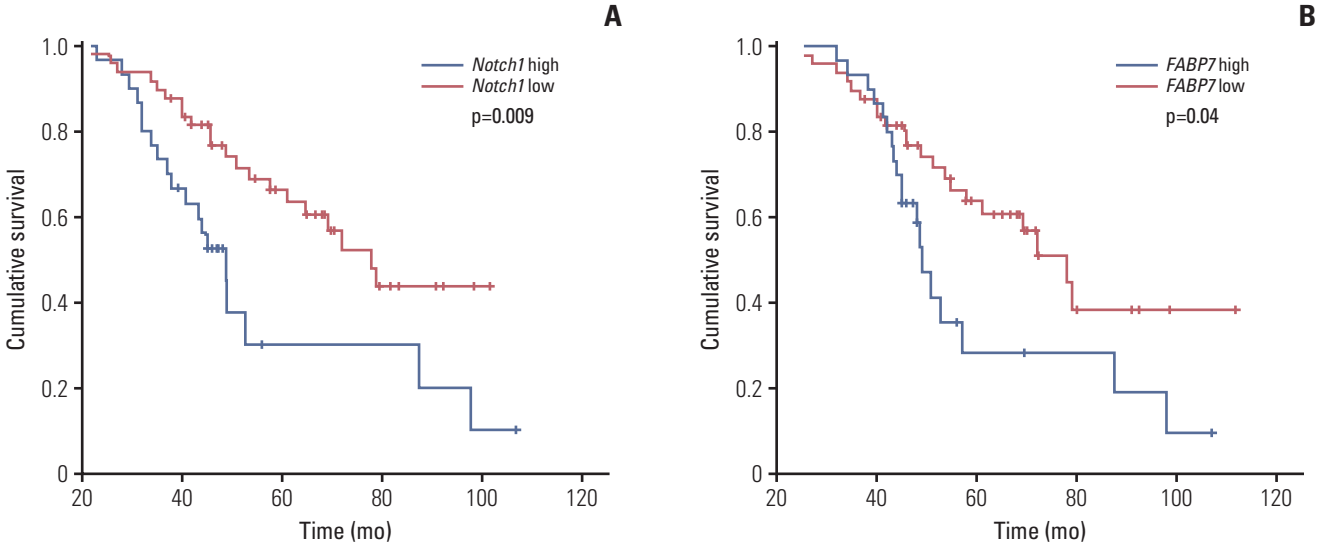

Fig. 2. Kaplan-Meier analysis of recurrence-free survival (RFS) in patients with tracheobronchial adenoid cystic carcinoma. (A) RFS in patients with *Notch1* low versus high expression. (B) RFS in patients with *FABP7* low versus high expression.

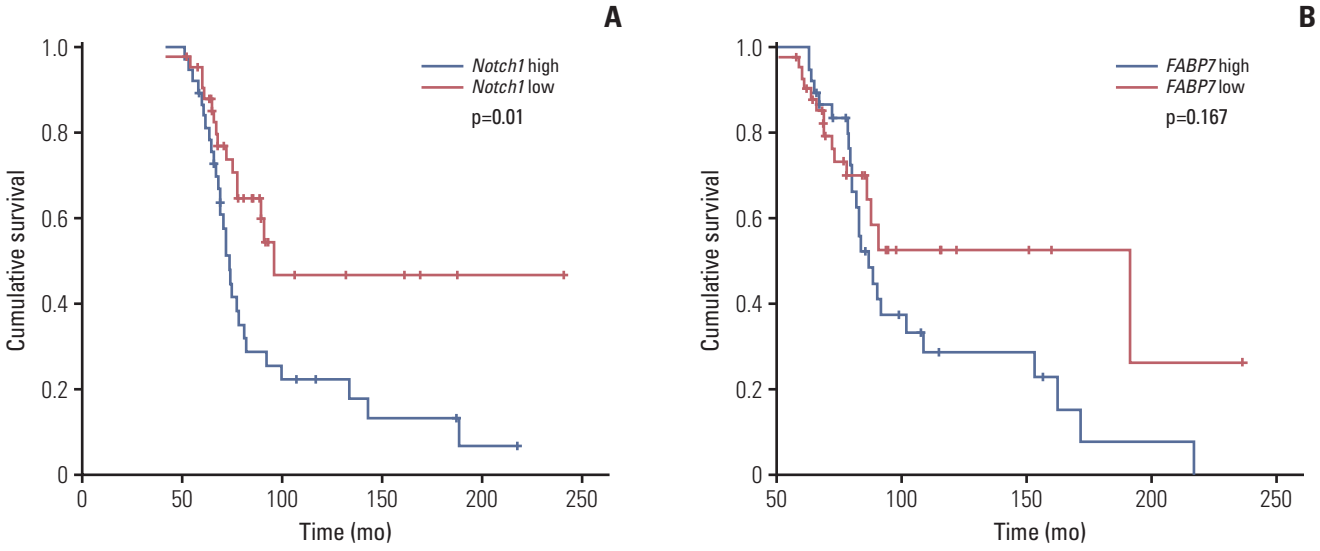

Fig. 3. Kaplan-Meier analysis of overall survival (OS) in patients with tracheobronchial adenoid cystic carcinoma. (A) OS in patients with *Notch1* low versus high expression. (B) OS in patients with *FABP7* low versus high expression.

FABP7 overexpression (p=0.048) (Table 2). In multivariate analysis for OS, the four variables that remained independently substantially associated with OS were positive resection margin (p=0.026), solid pattern (p=0.023), without postoperative therapy (p=0.021), and *Notch1* overexpression (p=0.018).

4. Prognostic prediction using combined *Notch1* and *FABP7* staining

We divided 368 patients into four groups according to the expression of *Notch1* and *FABP7*: $Notch1^{high}FABP7^{high}$ (n=100), $Notch1^{low}FABP7^{low}$ (n=89), $Notch1^{high}FABP7^{low}$ (n=96), and

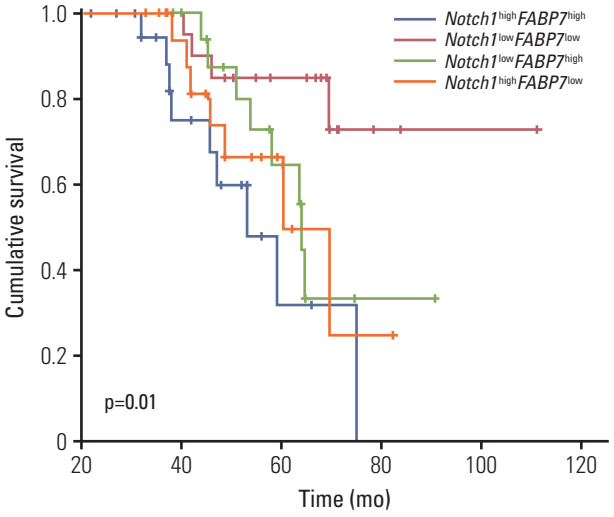

Fig. 4. Kaplan-Meier analysis of recurrence-free survival in patients with tracheobronchial adenoid cystic carcinoma stratified according to the expression of both *Notch1* and *FABP7*.

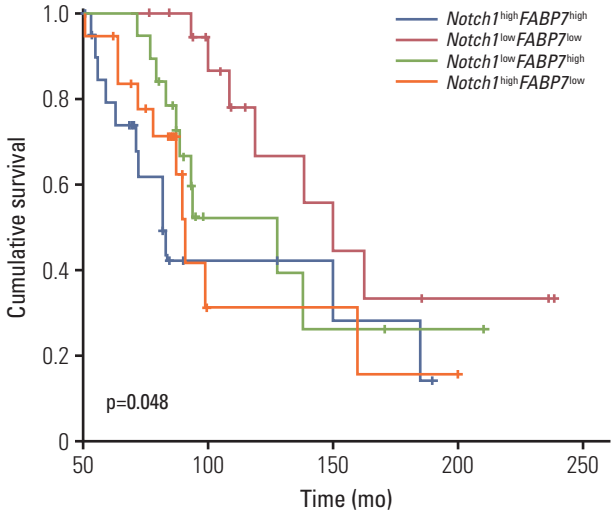

Fig. 5. Kaplan-Meier analysis of overall survival in patients with tracheobronchial adenoid cystic carcinoma stratified according to the expression of both *Notch1* and *FABP7*.

Notch1^{low}*FABP7*^{high} (n=83). Survival curves were generated by Kaplan-Meier method and differences between these four groups were compared by log-rank test. The results showed that patients with high expression of both *Notch1* and *FABP7* had significantly shorter RFS (p=0.01) (Fig. 4) and OS (p=0.048) (Fig. 5). In multivariate analysis, patients with high expression of both *Notch1* and *FABP7* (*Notch1*^{high} *FABP7*^{high}) had significantly shorter RFS (p=0.036; HR, 1.37; 95% CI, 1.12 to 1.89) (Table 2), but not OS (p=0.164; HR, 1.32; 95% CI, 1.12 to 1.89) (Table 3).

Discussion

Primary tumor of the upper airway is relatively rare. ACC is the second most frequent histologic type of tracheal tumor. ACC often develops in a relatively major airway between the trachea and the lobular bronchus [18]. Several poor prognostic factors of recurrence and metastasis have been reported. However, these prognostic factors remain uncertain [19,20].

In this study, we investigated the prognostic significance of *Notch1* and *FABP7* expression in patients with resected tracheobronchial ACC. Our results showed that the expression of *Notch1* and *FABP7* was significantly associated with shorter RFS in primary tracheobronchial ACC. In multivariable analysis, *Notch1* was an independent factor for both worse RFS and both OS. *FABP7* was only associated with shorter RFS. Overexpression of both *Notch1* and *FABP7* were associated with the worst outcome. Absence of expression of *Notch1* and *FABP7* identified a best prognostic group, whereas expression of one of these two markers conferred an intermediate prognosis.

In this study analyzing 368 patients with primary tracheobronchial ACC, 139 patients (37.8%) showed *Notch1* overexpression and 141 patients (38.3%) showed *FABP7* overexpression. The solid subtype was the most undifferentiated form in ACC [2]. Our study showed that *Notch1* and *FABP7* overexpression was associated with more solid subtype.

The Notch pathway is involved in maintenance of stem cells, cell proliferation, and angiogenesis [21]. Whole exome sequencing of ACC samples showed Notch pathway alterations occurred in 11% to 29% of patients [22,23]. Ferrarotto et al. [24] found that majority of *Notch1* mutations in ACC were activating. The association between *Notch1* mutation and solid subtype suggests that *Notch1* drives the pro-metastatic phenotype in ACC [25-27]. Canonical Notch signaling relies on a series of ligand-dependent proteolytic cleavages, and releases the Notch intracellular domain (NICD), which translocates to the nucleus and regulates transcription of targeted genes. Immunohistochemical staining of cleaved *Notch1* was sensitive to identify *Notch1* activation [24].

Notch1 and *SOX10* is essential for proliferation in ACC [28]. Knockdown of *Notch1*, *SOX10*, and downstream effector

FABP7 inhibited tumor formation, and induced cell death. *FABP7* belongs to a large family of hydrophobic proteins [29]. High expression of *FABP7* were observed in glioblastoma, breast cancer and renal cancer, and its expression was significantly associated with poor survival. *FABP7* is regulated differently by homeobox 1 (*EN1*) and *MYB* and was correlated with poor prognosis in salivary ACC. ACC with solid subtype had strong positive immunostaining of *FABP7* [16].

Current study shows the significant benefit of both RFS and OS from postoperative therapy by multivariate analysis. A high proportion (61.4%) of resected specimens had positive microscopic margins. Forty-eight point seven percent patients received postoperative radiotherapy and 12.3% patients received postoperative radiotherapy followed by chemotherapy. Both high expression of *Notch1* and *FABP7* showed a trend towards poor RFS. Combinatorial high expression of *Notch1* and *FABP7* was able to distinguish patients with worse prognosis regarding recurrence. Therefore, *Notch1* and *FABP7* may be useful for identifying patients with a high risk of recurrence who may benefit from postoperative therapy. Our findings clearly indicate *Notch1* and *FABP7* are independent prognostic factors of survival time and clinical indication of postoperative therapy especially radiotherapy in ACC of trachea and bronchus.

In conclusion, this study demonstrated the expression of *Notch1* and *FABP7* in a fraction of primary ACC of trachea and bronchus. Patients with high expression of *Notch1* and *FABP7* showed a significantly increased risk of recurrence and disease related mortality. Further prospective study with internal and external validation is necessary.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (http://www.e-crt.org).

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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