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Original Article

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VEGF and Ki-67 Overexpression in Predicting Poor Overall Survival in Adenoid Cystic Carcinoma

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Purpose

The purpose of this study was to evaluate potential prognostic factors in patients with adenoid cystic carcinoma (ACC).

Materials and Methods

A total of 68 patients who underwent curative surgery and had available tissue were enrolled in this study. Their medical records and pathologic slides were reviewed and immunohistochemistry for basic fibroblast growth factor, fibroblast growth factor receptor (FGFR) 2, FGFR3, c-kit, Myb proto-oncogene protein, platelet-derived growth factor receptor beta, vascular endothelial growth factor (VEGF), and Ki-67 was performed. Univariate and multivariate analysis was performed for determination of disease-free survival (DFS) and overall survival (OS).

Results

In univariate analyses, primary site of nasal cavity and paranasal sinus (p=0.022) and Ki-67 expression of more than 7% (p=0.001) were statistically significant factors for poor DFS. Regarding OS, perineural invasion (p=0.032), high expression of VEGF (p=0.033), and high expression of Ki-67 (p=0.007) were poor prognostic factors. In multivariate analyses, primary site of nasal cavity and paranasal sinus (p=0.028) and high expression of Ki-67 (p=0.004) were independent risk factors for poor DFS, and high expression of VEGF (p=0.011) and Ki-67 (p=0.011) showed independent association with poor OS.

Conclusion

High expression of VEGF and Ki-67 were independent poor prognostic factors for OS in ACC.

Key words
Adenoid cystic carcinoma, Immunohistochemistry, Vascular endothelial growth factor, Ki-67, Prognosis

Introduction

Adenoid cystic carcinoma (ACC) is uncommon malignancies, which commonly arise in the salivary glands [1], although nearly half of ACC occurs in other glandular areas, particularly in the nasal cavity and paranasal areas. They also arise in the tongue and the minor salivary glands [2,3], and, very rarely, originate at other sites, such as the external auditory canal, trachea, lung, breast, and Bartholin's glands [2].

ACC is characterized by unpredictable growth and extensive perineural invasion. Typically the natural course of ACC is slow; however, local recurrence and hematogenous spread to the lungs often occur during the course of the disease [4,5]. Despite the indolent growth pattern, once metastatic disease is present, disease progression becomes more rapid; one-third of patients die within 2 years of developing multiple metastases [6]. Treatment remains limited to surgery and radiation, and no systemic chemotherapeutic agent has been proven to be effective [7].

Due to its rarity, the molecular biology of ACC has not been well-described [8]. Clinical stage and solid histologic type were poor prognostic indicators of survival in several studies [9,10]. In immunohistochemistry, expression of Myb proto-oncogene protein (MYB), c-kit, vascular endothelial growth factor (VEGF), p53, and Ki-67 are known poor prognostic factors of ACC; however, the relationship between these markers and survival of patients is not well-known [9,11-15]. In addition, there are many potential biomarkers whose significance has not yet been determined.

A previous study reported that fibroblast growth factor (FGF), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor beta (PDGFR-beta) represented DNA copy number gain in ACC by microarray-based comparative genomic hybridization [16]. However, there is insufficient information regarding the immunohistochemical results of these markers.

The aim of this study was to evaluate the prognostic value of potential biomarkers related to ACC.

Materials and Methods

1. Patients

The medical records of 188 patients diagnosed with ACC at Seoul National University Hospital between 1990 and 2012 were reviewed. Among these patients, 68 patients who underwent curative surgery and had available tissue were enrolled in this study.

This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB approval No. H-1109-114-379). Demographics, clinical and pathologic data, and treatment-related factors with regard to recurrence and patient survival were collected from medical records. Sixteen patients (24%) were followed up until death, and the median follow-up period was 68 months (range, 4 to 263 months).

2. Immunohistochemistry

Core tissues (2 mm in diameter) were taken from representative formalin-fixed paraffin-embedded tissue blocks and tissue microarrays were constructed for further immunohistochemical analysis. The antibodies used were basic FGF (bFGF; 1:100, Calbiochem, San Diego, CA), FGFR2 (H2263-M01, 1:3,000, Abnova, Taipei, Taiwan), FGFR3 (SC-13121, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA), c-kit (A2502, 1:200, DAKO, Carpinteria, CA), MYB (#1792-1, 1:80, Epitomics, Burlingame, CA), PDGFR-beta (SC-713, 1:100, Santa Cruz Biotechnology), VEGF (SC-7269, 1:1,000, Santa Cruz Biotechnology), and Ki-67 (M7240, 1:100, DAKO).

Immunostaining was evaluated semi-quantitatively for intensity (0, negative; +1, weak positive; +2, moderate positive; and +3, strong positive) and extent (0, 0%; +1, 1%-25%; +2, 26%-50%; +3, 51%-75%; and +4, 76%-100%) by two experienced pathologists (S.J.N., Y.K.J.); normal salivary gland samples were used as controls. Score was determined by multiplying extent and intensity. Positivity of expression was defined as a score of 4 or more, and high expression was defined as a score of 8 or more. Conversely, c-kit was graded only by extent (0 to +4) because its expression was strong enough in all positive specimens, and positivity was defined as a grade of 2 or more [17].

The immunostained slides for Ki-67 were submitted to virtual microscope scanning under high-power magnification (×200) using ScanScope CS2 eSlide (Aperio Technologies, Vista, CA). Estimation of Ki-67 expression was based on the proportion of positive cells in all tumor cells using the nuclear v9 algorithm of ImageScope software (Aperio Technologies). Ki-67 was considered positive when the tumor cells showed strong nuclear staining intensity.

3. Statistical analysis

Statistical analyses of categorical variables were performed using Pearson's χ^2 test or Fisher exact probability test where appropriate. The median duration of survival was calculated using the Kaplan-Meier method, and comparisons between groups were made using the log-rank tests. To determine the important prognostic factors, Cox proportional hazards

Table 1. Clinicopathological features and univariate Cox regression analysis of 68 patients

Characteristic	No. (%)	DFS		OS	
		HR	p-value	HR	p-value
Sex					
Male	26 (38.2)	1		1	0.881
Female	42 (61.8)	0.78	0.463	1.09	
Age (yr)					
≤ 45	17 (25.0)	1		1	0.658
> 45	51 (75.0)	1.55	0.251	1.30	
Primary site					
Salivary gland	36 (52.9)	1		1	
Nasal cavity, paranasal sinus	16 (23.5)	2.34	0.022	1.61	0.420
Tongue, oral cavity	7 (10.3)	2.01	0.146	0.81	0.841
Lung, trachea	4 (5.9)	0.33	0.280	1.44	0.738
Others[a]	5 (7.4)	1.00	0.995	1.01	0.994
Local treatment					
Operation with PORT	49 (72.1)	0.96	0.919	2.16	0.311
Operation without PORT	19 (27.9)	1		1	
Any chemotherapy					
Yes	11 (16.2)	-[b]	-[b]	2.32	0.116
No	57 (83.8)	-		1	
Perineural invasion					
Yes	23 (33.8)	1.26	0.501	3.05	0.032
No	45 (66.2)	1		1	
Resection margin					
Positive	39 (57.4)	0.97	0.913	1.49	0.456
Negative	29 (42.6)	1		1	

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; PORT, postoperative radiotherapy. [a]Bartholin's gland, external auditory canal, and lacrimal gland, [b]There was no perioperative chemotherapy.

regression models were used in univariate and multivariate analyses. A two-sided value of $p < 0.05$ was considered statistically significant. All analyses were performed using SPSS for Windows ver. 20.0 (IBM Co., Armonk, NY).

Results

1. Clinicopathological data

Of the 68 patients, 26 patients were male and 42 were female, with median age of 55 years (range, 26 to 84 years). Primary sites were major salivary glands in 36 cases (53%), nasal cavity and paranasal sinuses in 16 (24%), oral cavity and tongue in seven (10%), lung and trachea in four (6%), and other sites in five (7%). Other sites included the lacrimal gland, Bartholin's gland, and external auditory canal. Twenty-three patients (34%) had perineural invasion and 39 (57%) had involvement of resection margins. Local recurrence was detected in 21 patients (31%), regional recurrence in three (4%), distant metastases in 35 (51%), and any kind of recurrence in 39 (57%). Of patients with distant metastases, 32 patients (47%) had lung metastasis, nine (13%) had liver metastasis, six (9%) had bone metastasis, and two (3%) had central nervous system metastasis.

Analysis of patients was based on clinicopathological factors, including sex, age, primary site, local treatment modality including postoperative radiotherapy, systemic chemotherapy, perineural invasion, and positive resection margins. Survival analysis was performed in each subgroup using the Cox proportional hazards regression model. Among these clinicopathological variables, primary site of nasal cavity and paranasal sinus was a poor prognostic factor for disease-free survival (DFS; hazard ratio [HR], 2.34; 95% confidence interval [CI], 1.13 to 4.84; p=0.022). Perineural invasion was the only significant prognostic factor of poor overall survival (OS; HR, 3.05; 95% CI, 1.10 to 8.44; p=0.032) (Table 1).

Table 2. Positive expression of molecular markers and univariate Cox regression analysis of disease-free survival and overall survival in adenoid cystic carcinoma

Variable	Score	No. (%)	DFS			OS		
			HR	95% CI	p-value	HR	95% CI	p-value
bFGF	< 4	20 (29.4)	1			1		
	≥ 4	48 (70.6)	0.76	0.39 to 1.49	0.424	1.25	0.35 to 4.47	0.737
c-kit	< 2	16 (23.5)	1			1		
	≥ 2	52 (76.5)	0.94	0.46 to 1.94	0.872	0.75	0.23 to 2.43	0.632
FGFR2	< 4	62 (91.2)	1			1		
	≥ 4	6 (8.8)	1.45	0.51 to 4.10	0.488	1.86	0.40 to 8.68	0.432
FGFR3	< 4	64 (94.1)	1			1		
	≥ 4	4 (5.9)	3.26	1.13 to 9.44	0.029	0.94	0.12 to 7.22	0.952
MYB	< 4	44 (64.7)	1			1		
	≥ 4	24 (35.3)	1.02	0.51 to 2.02	0.959	1.14	0.36 to 3.62	0.831
PDGFR-beta	< 4	25 (36.8)	1			1		
	≥ 4	43 (63.2)	1.10	0.57 to 2.13	0.770	2.43	0.68 to 8.62	0.171
VEGF	< 4	6 (8.8)	1			1		
	≥ 4	62 (91.2)	1.96	0.60 to 6.40	0.264	2.53	0.33 to 19.39	0.371

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; bFGF, basic fibroblast growth factor; FGFR2, fibroblast growth factor receptor 2; FGFR3, fibroblast growth factor receptor 3; MYB, Myb proto-oncogene protein; PDGFR-beta, platelet-derived growth factor receptor beta; VEGF, vascular endothelial growth factor.

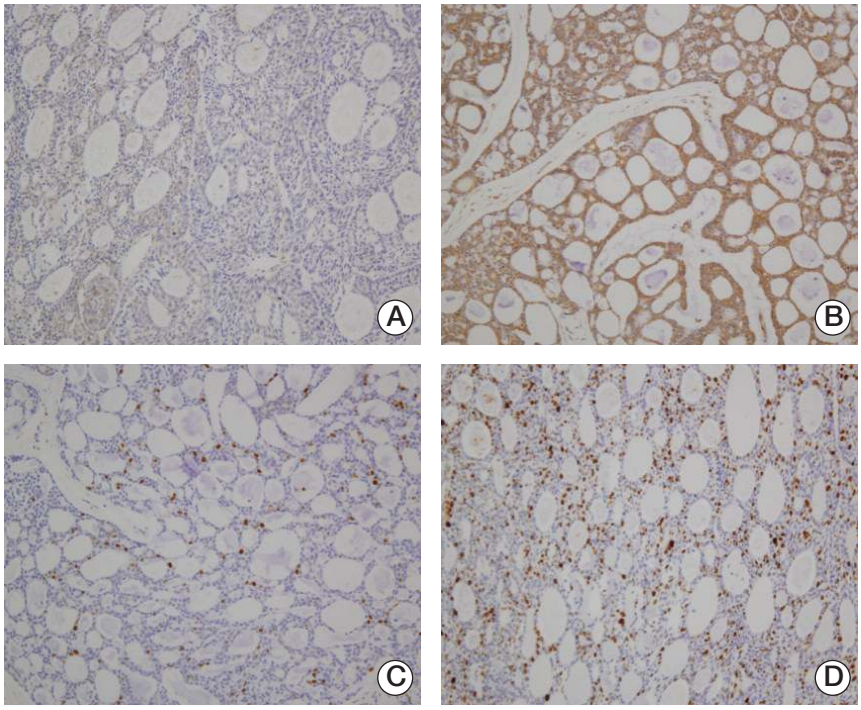

Fig. 1. Immunohistochemical staining of adenoid cystic carcinoma. (A) Low expression of vascular endothelial growth factor (VEGF). (B) High expression of VEGF. (C) Low expression of Ki-67. (D) High expression of Ki-67 (A-D, ×200).

Table 3. High expression of molecular markers and univariate Cox regression analysis of disease-free survival and overall survival in adenoid cystic carcinoma

Variable	Score	No. (%)	DFS			OS		
			HR	95% CI	p-value	HR	95% CI	p-value
bFGF	< 8	42 (61.8)	1			1		
	≥ 8	26 (38.2)	0.85	0.44 to 1.62	0.618	1.25	0.44 to 3.51	0.673
c-kit	< 4	51 (75.0)	1			1		
	≥ 4	17 (25.0)	1.12	0.52 to 2.45	0.771	1.13	0.25 to 5.13	0.876
FGFR2	< 8	67 (98.5)	1			1		
	≥ 8	1 (1.5)	15.98	1.79 to 143.04	0.013	62.50	3.91 to > 99.99	0.003
FGFR3	< 8	67 (98.5)	1			1		
	≥ 8	1 (1.5)	4.09	0.54 to 31.10	0.174	0.05	< 0.01 to > 99.99	0.883
MYB	< 8	60 (88.2)	1			1		
	≥ 8	8 (11.8)	1.13	0.40 to 3.19	0.820	0.04	< 0.01 to 44.54	0.370
PDGFR-beta	< 8	55 (80.9)	1			1		
	≥ 8	13 (19.1)	1.14	0.52 to 2.49	0.736	2.08	0.70 to 6.20	0.189
VEGF	< 8	29 (42.6)	1			1		
	≥ 8	39 (57.4)	1.31	0.69 to 2.48	0.403	3.45	1.11 to 10.71	0.033
Ki-67	< 7%	50 (73.5)	1			1		
	≥ 7%	18 (26.5)	3.25	1.58 to 6.68	0.001	4.47	1.52 to 13.18	0.007

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; bFGF, basic fibroblast growth factor; FGFR2, fibroblast growth factor receptor 2; FGFR3, fibroblast growth factor receptor 3; MYB, Myb proto-oncogene protein; PDGFR-beta, platelet-derived growth factor receptor beta; VEGF, vascular endothelial growth factor.

2. Immunohistochemical data

In immunohistochemistry, overexpression of bFGF was observed in 48 patients (71%), c-kit in 52 (77%), FGFR2 in six (9%), FGFR3 in four (6%), MYB in 24 (35%), PDGFR-beta in 43 (63%), and VEGF in 62 (91%). The median Ki-67 value was 4% (range, 0% to 33%) (Table 2, Fig. 1).

In univariate analysis, positive expression with a score of 4 or more for bFGF, FGFR2, MYB, PDGFR-beta, and VEGF and a score of 2 or more for c-kit did not show correlation with DFS and OS (Table 2). On the other hand, high expression with a score of 8 or more for VEGF was a poor prognostic factor for OS (HR, 3.45; 95% CI, 1.11 to 10.71; p=0.033), and Ki-67 expression of more than 7% was significant as a poor prognostic factor for both DFS (HR, 3.25; 95% CI, 1.58 to 6.68; p=0.001) and OS (HR, 4.47; 95% CI, 1.52 to 13.18; p=0.007) (Table 3, Figs. 2, 3A and B). Although a score of 4 or more for FGFR3 and a score of 8 or more for FGFR2 expression also showed a significant p-value, due to the few patients with expression, further validation of the prognostic values is needed in a larger sample size.

In multivariate analysis, primary site of nasal cavity and paranasal sinus (HR, 2.21; 95% CI, 1.09 to 4.50; p=0.028) and Ki-67 expression (HR, 3.05; 95% CI, 1.43 to 6.54; p=0.004) were independent risk factors for poor DFS. High expression of VEGF (HR, 5.44; 95% CI, 1.48 to 19.98; p=0.011) and Ki-67 (HR, 4.83; 95% CI, 1.44 to 16.21; p=0.011) were independently significant factors of poor OS. However, perineural invasion was not statistically significant for OS (HR, 2.90; 95% CI, 1.00 to 8.41; p=0.051) (Table 4). Conduct of further studies to determine relationship between perineural invasion and OS will be needed. Patients who had both high expression of VEGF and Ki-67 expression of more than 7% showed poorer OS than patients who had high expression of only one of the two proteins (HR, 8.58; 95% CI, 1.47 to 50.01; p=0.017) (Fig. 3C).

Discussion

The goal of the current study was to identify prognostic markers of ACC by evaluating the relationship between clinicopathological and immunohistochemical data and DFS and OS. The markers bFGF, c-kit, FGFR2, FGFR3, MYB, PDGFR-beta, VEGF, and Ki-67 were evaluated by immunohistochemistry. In univariate analysis, nasal cavity and paranasal sinus as primary site and Ki-67 expression of more than 7% were risk factors for poor DFS, and presence of perineural

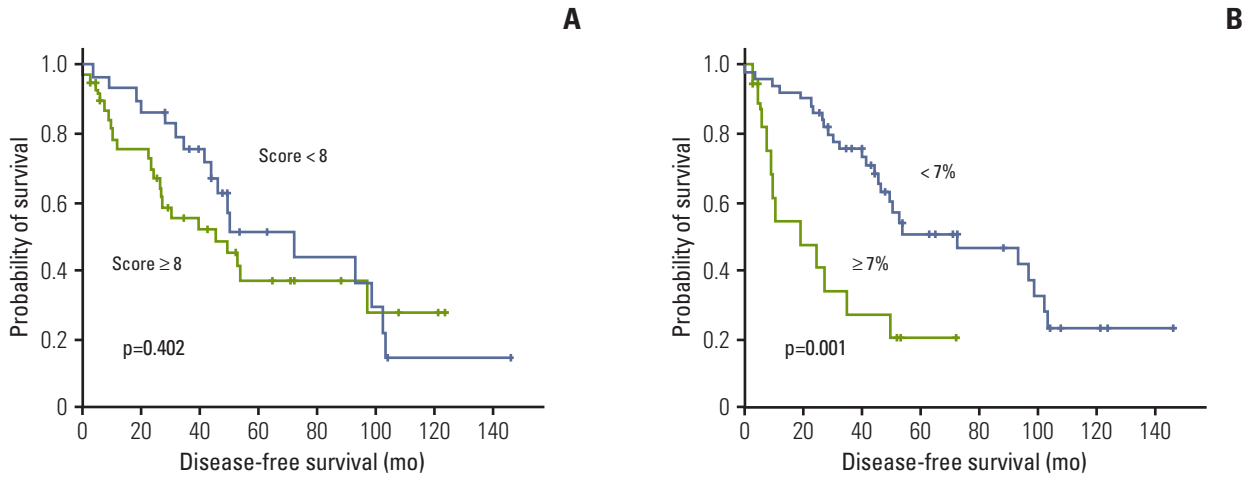

Fig. 2. Kaplan-Meier curve for disease-free survival by vascular endothelial growth factor expression (A) and Ki-67 expression (B). Comparisons were made using the log-rank test.

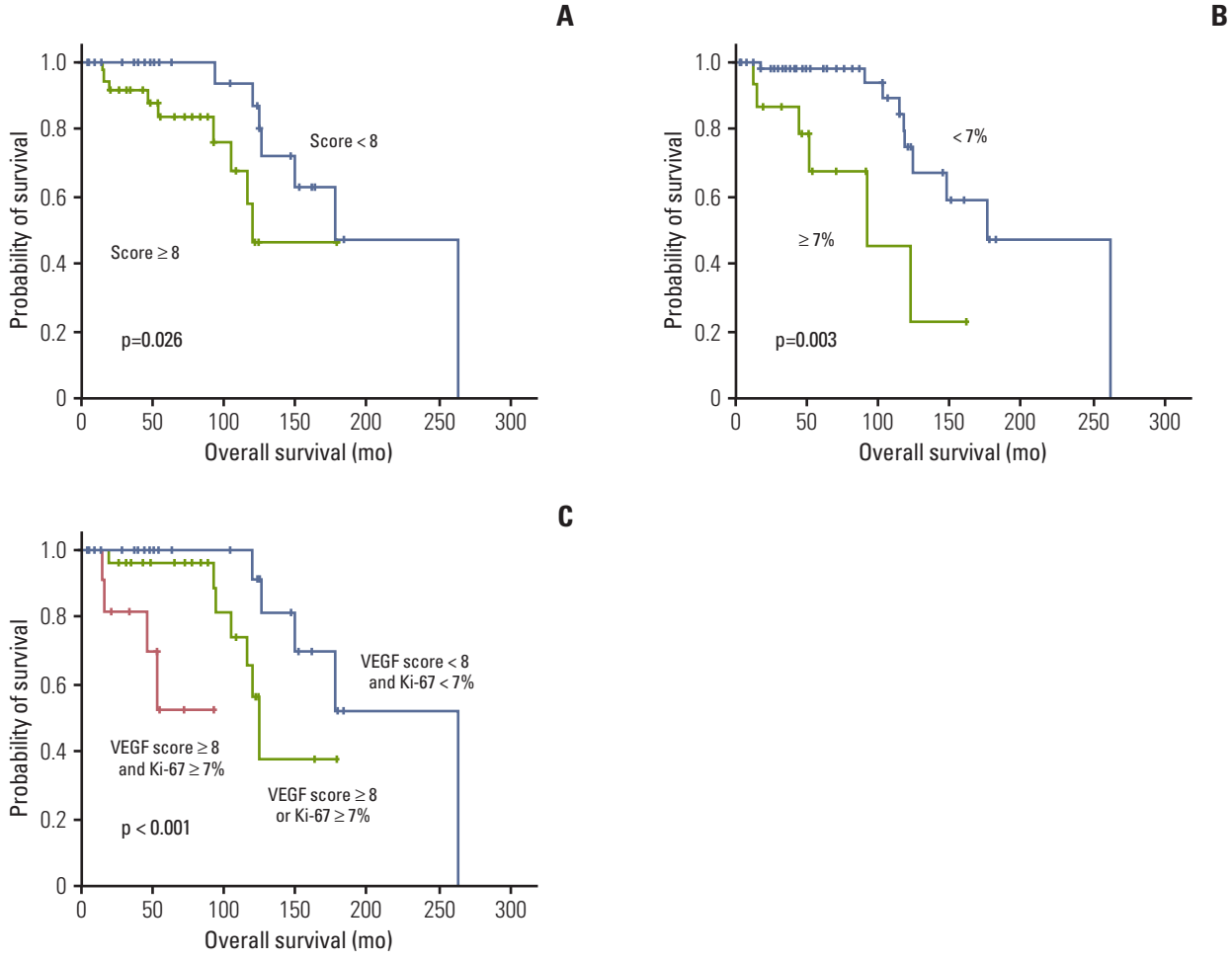

Fig. 3. Kaplan-Meier curve for overall survival by vascular endothelial growth factor (VEGF) expression (A), Ki-67 expression (B), and VEGF and/or Ki-67 expression (C). Comparisons were made using the log-rank test.

Table 4. Multivariate Cox regression analysis of disease-free survival and overall survival in adenoid cystic carcinoma

Variable	Category	HR	95% CI	p-value
DFS				
Nasal area[a)]	No	1		
	Yes	2.21	1.09 to 4.50	0.028
Perineural invasion	No	1		
	Yes	1.29	0.65 to 2.55	0.460
Resection margin	No	1		
	Yes	0.84	0.39 to 1.78	0.641
PORT	No	1		
	Yes	1.09	0.47 to 2.52	0.837
VEGF	< 8	1		
	≥ 8	1.31	0.67 to 2.56	0.435
Ki-67	< 7%	1		
	≥ 7%	3.05	1.43 to 6.54	0.004
OS				
Perineural invasion	No	1		
	Yes	2.90	1.00 to 8.41	0.051
VEGF	< 8	1		
	≥ 8	5.44	1.48 to 19.98	0.011
Ki-67	< 7%	1		
	≥ 7%	4.83	1.44 to 16.21	0.011

HR, hazard ratio; CI, confidence interval; DFS, disease-free survival; PORT, postoperative radiotherapy; VEGF, vascular endothelial growth factor; OS, overall survival. [a)]Primary sites including nasal cavity and paranasal sinus.

invasion and high expression of VEGF and Ki-67 were significant prognostic factors for poor OS. Among them, high expression of VEGF and Ki-67 were independently significant prognostic factors for OS in multivariate analyses.

ACC has diverse primary sites and there are varying levels of difficulty of operation according to primary sites. Ciccolallo et al. [18], who analyzed survival of ACC in a large population using the EUROCARE database, showed that ACC originating from nasal cavity, pharynx, and larynx have poorer 5-year survival rate than oral cavity and major salivary glands. In the current study, although no significant difference of OS was observed among primary sites, nasal cavity and paranasal sinus were the sites showing poor DFS.

In the current study, perineural invasion was observed in 23 patients (34%), and it was the only significant prognostic factor for OS among the clinicopathological values evaluated. Its importance as a poor prognostic factor is well-established in the literature [5,9,19]. In the study reported by Garden et al. [19], in addition to the presence of perineural invasion, positive resection margin was an adverse prognostic factor in terms of local control, which was not the case in this study.

VEGF, a critical factor in tumor angiogenesis, has been widely assessed in various types of cancer. Association of VEGF expression with poor prognosis of ACC has been reported in a few studies [12,13,20]. In an analysis with 29 patients of salivary gland carcinomas, Lim et al. [13] reported a significant association of high expression of VEGF with poor OS. However, only 15 patients were ACC patients and others were mucoepidermoid carcinomas. Li et al. [20] reported correlation of high VEGF expression with tumor stage in 55 patients of salivary ACCs, but not with local recurrence and survival rate. High expression of VEGF was an independently important factor for poor OS in the current study. Although VEGF expression showed no statistically significant difference for DFS, it showed a poor trend in the Kaplan-Meier curve. Conduct of further studies with a large population will be needed in order to verify correlation of VEGF expression with poor DFS.

Ki-67 is associated with cellular proliferation in tumor progression. Several studies identified high expression of Ki-67 as a negative prognostic factor in salivary gland carcinomas [13,14,21]. Nordgard et al. [21], who analyzed 44 patients diagnosed with ACCs, reported a significant difference of poor DFS with Ki-67 expression of more than 4%. Lim et al. [13], who analyzed survival of 29 patients, 15 of salivary ACCs and 14 of mucopeidermoid carcinomas, reported correlation between high expression of Ki-67 and poor OS. In addition, Ettl et al. [14] demonstrated relationship between Ki-67 expression and poor OS in 101 patients of salivary gland carcinomas. However, that population only included 25 ACC patients. In the current study, Ki-67 was the strongest prognostic marker for both poor DFS and OS in ACC. This is the largest study to date composed only of ACC patients, and proves significance of Ki-67 expression. In our analysis a cut-off value of 7% showed the greatest significance in OS.

One of the most studied biomarkers of ACC is the translocation between *MYB* oncogene and *NFIB* translocation factor. Approximately 50% of patients have a *MYB-NFIB* translocation, and these patients tend to have a higher risk for local relapse [22]. MYB over-expression has been associated with *MYB-NFIB* translocation, although its relationship with ACC prognosis was uncertain [23]. In our study, MYB expression was not a significant factor in predicting poor DFS or OS. Another biomarker, c-kit, has been the subject of several research studies in ACC. In a recent study, c-kit expression showed correlation with clinical stage, perineural invasion, locoregional recurrence, and distant metastases; however, there was little data regarding the relationship between c-kit expression and OS [11]. c-kit expression was not a significant prognostic factor of DFS and OS in the current study.

PDGFR-beta expression is known to be a poor prognostic

factor in breast cancer [24,25]. Similarly, DNA copy numbers of PDGFR-beta on chromosome 5 are increased in ACC and it is a possible factor contributing to the progression of ACC [16]. However, the relationship between PDGFR-beta expression and the prognosis of ACC has not been previously reported. The current study showed that high expression of PDGFR-beta was not significant as a prognostic factor in ACC. DNA copy number gain of FGF and FGFR in ACC was also reported in an earlier study [16]; however, there was almost no data on the immunohistochemical expression of FGF and FGFR which was investigated in this study. bFGF expression of more than 70% was observed, however expression of FGFR2 and FGFR3 was rare. Among them, bFGF had no significant value as prognostic markers, and conduct of further studies on FGFR2 and FGFR3 will be needed in order to provide patients with practical recommendations.

Most studies conducted in the past analyzed the utility of immunohistochemical markers with respect to ACC invasiveness and recurrence. However, the current study was conducted in a large population and investigated the correlation between several immunohistochemical markers and survival, which is more critical for ACC prognosis. We acknowledge that there are some limitations to this study, including the lack of validating the immunohistochemical cut-off values used, and the fact that this is a retrospective study.

Conclusion

In conclusion, high expression of VEGF and high expression of Ki-67 are independent prognostic factors of poor OS in ACC. Therefore, more aggressive and differentiated treatment should be provided to patients who show high expression of these markers. Conduct of further prospective studies in larger populations will be necessary in order to confirm these prognostic factors and to elucidate appropriate treatment modalities.

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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