Intestinal Stem Cell Marker, ASCL2 Is a Novel Prognostic Predictor in Esophageal Adenocarcinoma

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Abstract

Purpose: Stem cell markers play a significant role in esophageal adenocarcinoma carcinogenesis via Barrett's esophagus; however, its utility as a prognostic biomarker has not been established.

Methods: We analyzed the immunohistochemical expression of Ascl2 and Lgr5 utilizing whole slides (WS; 35 cases) and tissue microarray (TMA; 64 cases of esophageal adenocarcinoma with adjacent normal squamous epithelium, Barrett's esophagus, and dysplasia). Two pathologists semi-quantitatively scored stained slides independently/blindly, and the results were correlated with clinicopathologic factors and outcomes.

Results: In WS, 51% and 57% expressed high Ascl2 and high Lgr5; in TMA, 69% and 88% expressed high Ascl2 and high Lgr5, respectively. In TMA, high Ascl2 and low Lgr5 expression significantly correlated to a higher number of involved lymph nodes (p=0.027 and p=0.0039), and Lgr5 expression significantly correlated to the pathological stage (p=0.0032). Kaplan-Meier analysis showed a negative impact of high Ascl2 expression on overall survival (OS; WS p=0.0168, TMA p=0.0276) as well as progression-free survival (PFS; WS p=0.000638, TMA p=0.0466), but not Lgr5. Multivariate Cox regression analysis revealed that Ascl2 expression is an independent prognostic factor for esophageal adenocarcinoma (OS; WS p=0.25, TMA p=0.011. PFS; WS p=0.012, TMA p=0.038). Analysis of the TCGA dataset showed that ASCL2 mRNA levels were correlated to nodal status but not overall survival.

Conclusion: Intestinal stem cell marker, Ascl2 is an independent prognostic factor in esophageal adenocarcinoma. High Ascl2 levels were associated with nodal status in both immunohistochemical and mRNA expression. These findings may contribute to the development of prognosis-based subclassification of esophageal adenocarcinoma.

Introduction

Esophageal adenocarcinoma (EAC) is one of the least studied cancers and is currently the sixth-leading cause of cancer death [1]. The epidemiology of EAC is striking in that it is the dominant histological type in the Western world, and its incidence is steadily continuing to increase [2]. One pathway to EAC involves gastroesophageal reflux disease resulting in Barrett's esophagus (BE), and subsequent EAC in 0.5%-1%. Dysplasia is commonly observed during this process [1]. BE is characterized by the replacement of normal squamous epithelium of the lower portion of the esophagus with metaplastic columnar epithelium and was long believed to be merely a defense mechanism to acidic refluxate. However, recent studies have given rise to at least four theories to explain the origin of BE and how BE is formed remains controversial to date [3]. Nonetheless, the fact remains that BE is the primary precursor lesion and a potent risk factor of EAC [4].

There has been extensive research in colorectal carcinoma, surrounding the roles of intestinal stem cells (ISCs) in its development and metastasis [5,6]. ISCs found at the base of intestinal crypts, expresses *Leucine-rich-repeat-containing G-protein-coupled receptor* (Lgr5), a molecular marker that sustains the self-renewing function of ISCs [7]. Recent studies demonstrated Lgr5 positive cells to make up a subpopulation of cancer stem cells (CSCs), proving the dual role of Lgr5 to regulate "stemness" in both healthy ISCs and

CSCs [8]. Lgr5 upregulation is observed in various cancers, including ISC-associated cancers such as colorectal and stomach carcinoma [9,10], and this upregulation leads to the activation of the Canonical Wnt signaling pathway [11]. Altered Wnt signaling plays a dominant role in BE progression [12], and its activation is shown to promote dysplastic change [13].

Gene profiling of Lgr5 positive ISCs led to the discovery of *Achaete scute-like* 2 (ASCL2), and transgenic manipulation of ASCL2 revealed that ISC fate relies on this expression [14]. ASCL2 is known to have another important function as a primary helix-loop-helix transcription factor and itself a downstream target of Wnt signaling [15], which is critical in the carcinogenesis and progression of EAC.

Considering that BE not only resembles the intestinal mucosa morphologically but also possesses an ISC population within [16,17], we hypothesized that key ISC markers, Lgr5 and Ascl2, have clinicopathological significance in EAC. We approached this using immunohistochemistry of whole slides (WS), tissue microarray (TMA) and validated it with TCGA expression data.

Patients And Methods

Patient selection

Patients diagnosed with EAC at University Health Network, Toronto, ON, Canada, between 2001 and 2011 were included in our study. Locally advanced as well as distant metastatic patients were enrolled. All methods were performed in accordance with relevant guidelines/regulations. University Health Network Research Ethics Board (CAPCR/UHN REB number 13-6551) approved this study, and all participants provided written informed consent to have their surgical specimens banked and used in future research. Clinicopathological information (age, gender, pathological stage, histological grade, neoadjuvant therapy, positive lymph nodes) were obtained from electronic hospital records, and the original Hematoxylin and Eosin (H&E) stained slides were reviewed by two pathologists.

WS preparation and TMA construction

Thirty-five cases were available for WS IHC. WS sections were cut at the 4-µm interval, and unstained slides were prepared for IHC.

Sixty-four cases were available for TMA block construction. After verification with H&E staining, representative tumor areas of up to three areas, 0.6 mm in diameter, were selected and deposited into a paraffin block with a tissue-array instrument (Beecher Instruments, Silver Springs, MD, USA). When applicable, normal squamous epithelium, BE, and glandular dysplasia were inserted adjacent to tumor samples. Consecutive 4-µm unstained TMA sections were cut and placed on slides for IHC analysis.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in an alcohol series. Blocking was performed using Hydrogen peroxide (3%, 10minutes). Antigen retrieval was performed in the Decloaker solution (pH9) using a Decloaking Chamber (Biocare Medical). The slides were incubated overnight at 4

degrees Celsius, with primary antibodies anti-Ascl2 (R&D systems, cat#AF6539, 1:200) and anti-Lgr5 (Abcam, cat#75850, 1:200). After washing with PBST, the slides were incubated with a secondary antibody for 60 minutes and then washed again with PBST. Diaminobenzidine (DAB) was used as a chromogen, and nuclear counterstaining was performed with hematoxylin. Then slides were dehydrated through graded alcohols, cleared in xylene and coverslipped. For negative control, we used PBS instead of the primary antibody.

Immunohistochemical analysis

IHC scores were evaluated by two pathologists independently, and both pathologists were blinded from clinical data. The scoring system was based on previously published literature [18,19]. Briefly, Ascl2 and Lgr5 were semiquantitatively scored for staining intensity (1-weak; 2-moderate; 3-strong) and the percentage of positive staining cells (0, <10%; 1, 11-30%; 2, 31-50%; 3, 51-80%; 4, >80%) [18,19]. All final IHC scores were calculated by multiplying the two factors with a cut-off value of 4 [18,19]. If scores for the two samples were discordant, the final score for the tumor was upgraded to the higher score.

Follow-up

For survival analysis, disease-free survival (DFS) and overall survival (OS) were calculated. Two patients who died within six months of surgery, due to post-surgical complications were excluded from both WS and TMA survival analysis.

Statistical analysis

All data were analyzed with R version 3.5.2 (The R Foundation for Statistical Computing, Vienna, Austria). Univariable and multivariable statistical analyses were performed to determine the association among Ascl2/Lgr5 expression levels, clinicopathological features, and time-to-event outcomes (OS and PFS). Differences in Ascl2/Lgr5 expression levels were assessed in univariable analysis using Fisher's exact tests on categorical variables and Wilcoxon-Mann-Whitney tests on continuous variables. Age, sex, histological grade, number/positivity of metastatic lymph nodes, pathological stage, and neoadjuvant therapy were considered as potential confounding factors. The Kaplan-Meier (KM) method and the Cox proportional hazards regression (Cox PH) were performed to investigate the association between Ascl2/Lgr5 expression levels and time-to-event outcomes. For KM, differences in survival curves were ascertained by the log-rank test. In Cox PH, both adjusted and unadjusted models were fitted. A p-value of less than 0.05 was considered statistically significant.

Bioinformatic analysis

In an attempt to validate the prognostic value of ASCL2 in EAC, mRNA and clinical profile data were downloaded from the online cBioPortal for Cancer Genomics (http://www.cbioportal.org/. date last accessed, January 29, 2020) [20,21]. Data from PanCancer Atlas was used [22]. cBioportal was also used to analyze the alteration frequency of ASCL2 mutations in EAC.

Results

Ascl2 and Lgr5 expression on whole slides

The clinicopathological characteristics of 35 cases in WS are summarized in Table 1. Briefly, the age range was 38 to 78 years old (mean age of 65.5 years), and males were the predominant sex (89%). 20% of the patients received neoadjuvant chemotherapy. The pathological stage was based on the 8th edition AJCC/UICC staging of cancers of the esophagus and esophagogastric junction [23].

Based on IHC scores, 18 cases (51%) were Ascl2^{high,} and 17 cases (49%) were Ascl2^{low}. Ascl2 was localized to the cytoplasm of tumor cells. Ascl2 expression tended to be correlated to histological grade (p=0.086) but did not have any significant correlation with any other clinicopathological factors (Table 1). For our study population (WS), the median overall survival (OS) time was 1.8 years (95% CI,0.7-7.4), and progression-free survival (PFS) time was 0.9 years (95% CI, 0.7-1.4). Ascl2 showed statistical significance in the KM analyses for OS and PFS, p=0.0168, and p=0.000638, respectively (Figure 1). Univariable Cox PH analyses showed Ascl2 significance in both OS and PFS (Table 2 and 3; OS HR=2.83, 95% CI=1.16-6.9, p=0.022; PFS HR=3.93, 95% CI=1.69-9.12, p=0.0014). In multivariate analysis adjusting for pathological stage, Ascl2 remained statistically significant for PFS (HR=3.13, 95% CI=1.28-7.65, p=0.012) but not for OS (HR=1.78, 95% CI=0.66-4.76, p=0.25) (Table 2 and 3).

Lgr5 was localized to the cytoplasm and membrane of tumor cells. Twenty cases (57%) were Lgr5^{high,} and 15 cases (43%) were Lgr5^{low}. Lgr5 was not significantly correlated to any of the clinicopathological factors (Table 1). Lgr5 did not show statistical significance in KM analyses for survival (OS: p=0.449, PFS: p=0.459; Data not shown). Univariable Cox PH analyses also failed to show Lgr5 significance in OS and PFS (Table 2 and 3: OS HR=0.74, 95% CI=0.31-1.77, p=0.5; PFS HR=0.75, 95% CI=0.34-1.66, p=0.48).

Ascl2 and Lgr5 expression on TMA slides

Sixty-four EAC patients were included in the TMA slides. Briefly, the age range was 38 to 86 years old (mean age of 64.6 years), and males were the predominant sex (84%). 31% of the patients received neoadjuvant chemotherapy (Table 4).

Twenty cases were Ascl2^{low,} and 44 cases were Ascl2^{high}. The representative picture of Ascl2^{high} is shown in Figure 2a. High Ascl2 expression was associated with a more significant number of positive lymph nodes (p=0.027). No association was observed between Ascl2 expression and age, sex, pathological stage, neoadjuvant therapy, or histological differentiation.
For the cases in TMA, the median overall survival (OS) time was two years (95% CI,1.4-3.2), and progression-free survival (PFS) time was 0.9 years (95% CI, 0.7-1.3). The median OS time for Ascl2^{high} was 1.4 years (95% CI, 0.9-2.3) compared to the much longer OS time for Ascl2^{low} at 5.2 years (95% CI, 2.6-8.2). The results of the KM analysis for OS is shown in Figure 3a (p=0.0276). Figure 3b shows the significant association of Ascl2 with PFS. The median PFS time for Ascl2^{high} was 0.8 years (95% CI, 0.5-1.3), compared to the 1.3 years for Ascl2^{low} (95% CI 1-8.2, p=0.0466). The results of the univariate and

multivariate analyses for OS (Table 5) and PFS (Table 6) support the results of KM analysis. Of note, multivariate analysis indicated that Ascl2 was an independent predictor of prognosis in line with gender and stage.

Fifty-six cases (88%) were Lgr5^{high}; 8 cases (13%) were Lgr5^{low}. Representative picture of Lgr5^{high} is shown in Figure 2b. 6 out of 8 Lgr5^{low} cases (75%) were pathological stage IV. Lgr5^{low} had significantly more positive lymph nodes compared to Lgr5^{high} (p=0.039). No other association was observed between Lgr5 expression and other clinicopathological factors. In KM analysis, Lgr5 was not a predictive factor of OS (Lgr5^{low} 1.8years 95% CI 0.7-2.8; Lgr5^{high} 2years 95% CI 0.7-7.4 p=0.393) nor PFS (Lgr5^{low} 0.9years 95% CI 0.5-1.4; Lgr5^{high} 1.2years 95%CI 0.5-14.7 p=0.62). Univariable Cox PH analyses also failed to show Lgr5 significance in OS and PFS (Table 5, 6: OS HR=1.27, 95% CI=0.52-3.09, p=0.59; PFS HR=1.69, 95% CI=0.67-4.21, p=0.26).

No significant correlation was seen between the expressions of Ascl2 and Lgr5 (p=0.42). 37 out of 64 cases (57.8%) were Ascl2^{high}/Lgr5^{high}; 19 cases (29.7%) Ascl2^{low}/Lgr5^{high}; 7 cases (10.9%) Ascl2^{high}/Lgr5^{low}; and one case (1.6%) Ascl2^{low}/Lgr5^{low}.

Ascl2 protein expression is significantly increased in esophageal adenocarcinoma

Adjacent 43 normal squamous epithelium, 22 BE, and six glandular dysplasia of the TMA patients were included in the TMA. EAC showed significantly increased Ascl2 expression compared to the normal squamous epithelium (p<0.001) and BE (p<0.001) and a trend towards higher expression compared to glandular dysplasia (p=0.0686, Figure 4a). Expression of Lgr5 in the normal squamous epithelium was significantly lower compared to the tumor (p<0.0001), BE (p<0.0001), and dysplasia (p<0.0001) but tumor, BE and dysplasia showed similar expression (Figure 4b).

Further validation of ASCL2 in TCGA datasets

Significance of Ascl2 protein expression in both WS and TMA prompted us to analyze the TCGA dataset. 86 cases of EAC revealed that ASCL2 mRNA was correlated with nodal status. ASCL2 mRNA was not significantly correlated to survival using KM analysis (Figure 5). No mutation in the ASCL2 gene was identified in the TCGA dataset, but two cases showed deep deletion of the ASCL2 gene.

Discussion

Here, we have reported the role of the ISC marker ASCL2 in determining EAC prognosis, which may also be responsible for EAC progression via the Wnt pathway. The ability to appropriately classify cancer to specific subtypes is an effective strategy that EAC currently lacks, and immunohistochemistry remains the most available measure to detect biomarkers to this day [24]. In this study, we used immunohistochemistry to locate two key stem cell markers, Ascl2 and Lgr5, and our results provided promising evidence that Ascl2 could potentially be used as a prognostic marker in EAC.

We examined the expression of Ascl2 using IHC in WS and TMA, as well as mRNA expression in TCGA datasets. The first important finding is that Ascl2 expression was significantly increased in EAC, when compared with normal squamous epithelium and BE but not with glandular dysplasia. EAC carcinogenesis is a multistep process, starting from gastroesophageal reflux, progressing to BE [25], and alteration in the Wnt pathway is implicated in the carcinogenesis of EAC, similar to other intestinal cancers [12]. ASCL2 is not only its direct transcriptional target but also the potent ISC fate determinator, working as a master regulator of Lgr5 positive ISCs [14]. Our results show a concordant increase in Ascl2 expression from squamous epithelium, BE, dysplasia to EAC, and implies an essential role of Ascl2 in the early development of EAC.

Patients with high Ascl2 expression showed worse survival in both WS and TMA, compared with the Ascl2^{low} group. According to our literature search, this is the first study to reveal the prognostic significance of Ascl2 in EAC. Of note, a high Ascl2 score had a significant impact on survival in line with gender (p=0.047) and stage (p<0.001), while other pathological factors such as histological grade and the number of positive nodes did not show a significant impact. Ascl2, a key player in tumor progression and metastasis, has been studied in other cancers, including colorectal cancer [26,27], consistently associated with poor prognosis. ASCL2 mRNA and protein using PCR assay and Western Blot Analysis in EAC cell lines have been studied by Zhao et al., which showed amplification and overexpression of ASCL2 [28]. Our bioinformatics analysis using TCGA data showed that ASCL2 mRNA was correlated to nodal status but not to OS nor PFS; thus, further research is necessary to understand the mechanism of Ascl2 protein overexpression in EAC, with a particular focus on the Wnt pathway.

ISC markers have been extensively studied in colorectal cancer, where ASCL2 is known to control the fate of ISCs and colon cancer progenitor cells by regulating "stemness" genes such as Lgr5 and controls self-renewal via R-spondin1/Wnt activation [15]. Further, ISCs impact the plasticity of epithelial-mesenchymal transition in colorectal cancer, via the expression of microRNA-200 [29]. Contrarily, the impact of ISCs on the carcinogenesis of EAC has been studied to a much lesser extent. Only one study examined ASCL2 mRNA and protein overexpression in EAC cell lines, in which cells with stem cell-like features overexpressed ASCL2 [28], but to our knowledge, this study is the first to assess Ascl2 IHC expression in EAC. Becker et al., found Lgr5 to be heterogeneously expressed in 24 cases of EAC using IHC, in which they concluded a high Lgr5 score was associated with worse survival [30]. Our study also revealed heterogeneous staining of Lgr5 in WS; however, our study showed different results where Lgr5 was not a significant prognostic marker in neither TMA nor WS. As sampling is limited in TMA, the propensity score was universally higher in TMA compared to WS consequently. Therefore, a direct comparison of the results is not feasible; however, this discordance may illustrate the difficulty in using Lgr5 as a prognostic marker. While the existence of Lgr5 stem cell-like cells in BE and EAC is mostly confirmed, its significance on cancer progression and metastasis remains to be determined with future studies.

Conclusion

The authors investigated ASCL2 as a potential biomarker of EAC based on known dual functions; (a) by promoting the Wnt signaling pathway and (b) regulating the ISCs located near the base of BE. We demonstrate a correlation between ASCL2 and OS; however, our study is limited by the relatively small number of patients and the lack of a prospective validation set. Further research is required to understand this complex pathway and molecular mechanism before it can be implemented in the clinical setting. Nonetheless, ASCL2 is a promising biomarker for predicting EAC survival and a key to understand the carcinogenesis and intestinal stem cells of EAC.

Declarations

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Tables

Table 1. Clinicopathological characteristics and ASCL2, Lgr5 expression (WS)

Covariate (TMA slides)	Full sample (n=35)	ASCL2^{low} (n=17)	ASCL2^{high} (n=18)	p-value	Lgr5^{low} (n=20)	Lgr5^{high} (n=15)	p-value
Age				0.69			0.93
Mean (sd)	65.5 (10.6)	66.5 (10)	64.5 (11.3)		65.5 (10.8)	65.5 (10.6)	
Median (Min,Max)	66 (38,78)	66 (38,78)	65.5 (41,78)		68 (38,78)	64 (41,78)	
Gender				0.34			1
female	4 (11)	3 (18)	1 (6)		2 (10)	2 (13)	
male	31 (89)	14 (82)	17 (94)		18 (90)	13 (87)	
Stage				0.18			1
Ⅰ/Ⅱ	6 (17)	4 (24)	2 (11)		3 (15)	3 (20)	
Ⅲ	20 (57)	11 (65)	9 (50)		12 (60)	8 (53)	
Ⅳ	9 (26)	2 (12)	7 (39)		5 (25)	4 (27)	
Neoadjuvant chemotherapy				0.4			0.67
No	28 (80)	15 (88)	13 (72)		15 (75)	13 (87)	
Yes	7 (20)	2 (12)	5 (28)		5 (25)	2 (13)	
Nodes positive				0.22			0.89
Mean (sd)	5.6 (8.3)	3.4 (3.2)	7.7 (10.9)		4.7 (5.4)	6.8 (11.2)	
Median (Min,Max)	4 (0,45)	3 (0,11)	4 (0,45)		4 (0,24)	4 (0,45)	
Nodes sampled				0.35			0.2
Mean (sd)	23 (11.9)	25.7 (12.8)	20.4 (10.7)		20.6 (10.1)	26.1 (13.6)	
Median (Min,Max)	20 (0,49)	21 (9,49)	19.5 (0,45)		18.5 (6,49)	21 (0,46)	
Histological grade				0.086			0.48
G1	1 (3)	1 (6)	0 (0)		0 (0)	1 (7)	
G2	20 (57)	12 (71)	8 (44)		11 (55)	9 (60)	
G3	14 (40)	4 (24)	10 (56)		9 (45)	5 (33)	

Table 2. Univariable and Multivariable analysis of overall survival (WS)

Univariable				Multivariable			
Covariate n=35 (22 events)	HR (95%CI)	p-value	Global p-value	Covariate n=62 (46 events)	HR (95%CI)	p-value	Global p-value
Age	1.01 (0.97,1.05)		0.69	ASCL2 score			0.25
Gender			1	low	Reference		
female	Reference			high	1.78 (0.66,4.76)		
male	3e+08 (0e+00,Inf)						
Histological grade			0.31				
G1	Reference						
G2	0.61 (0.08,4.85)	0.64		Stage			0.0025
G3	1.23 (0.15,9.89)	0.85		ⅠⅠ/Ⅱ	Reference		
				Ⅲ	2.18 (0.49,9.78)	0.31	
				Ⅳ	17.92 (2.81,114.28)	0.0023	
Stage			<0.001				
Ⅰ/Ⅱ	Reference						
Ⅲ	2.22 (0.49,9.93)	0.041					
Ⅳ	23.95 (3.93,146.05)	<0.001					
Node positive	1.07 (1.03,1.11)		0.001				
ASCL2 score			0.022				
low	Reference						
high	2.83 (1.16,6.9)						
Lgr5 score			0.5				
low	Reference						
high	0.74 (0.31,1.77)						

Table 3. Univariable and multivariable analysis of progression-free survival (WS)

Univariable				Multivariable			
Covariate n=35 (26 events)	HR (95%CI)	p-value	Global p-value	Covariate n=64 (50 events)	HR (95%CI)	p-value	Global p-value
Age	0.99 (0.96,1.03)		0.73	ASCL2 score			*0.012*
				low	Reference		
Gender			1	high	3.13 (1.28,7.65)		
female	Reference						
male	3.1e+0.8 (0e+00,Inf)			Stage			*0.035*
				Ⅰ/Ⅱ	Reference		
				Ⅲ	1.21 (0.34,4.3)	0.77	
Stage			*0.0045*	Ⅳ	5.4 (1.15,25.33)	*0.033*	
Ⅰ/Ⅱ	Reference						
Ⅲ	1.47 (0.42,5.08)	0.55					
Ⅳ	9.15 (1.98,42.27)	*0.0046*					
Node positive	1.05 (1.01,1.09)		*0.0065*				
ASCL2 score			*0.0014*				
low	Reference						
high	3.93 (1.69,9.12)						
Lgr5 score			0.48				
low	Reference						
high	0.75 (0.34,1.66)						

Table 4. Clinicopathological characteristics and ASCL2, Lgr5 expression (TMA)

Covariate (TMA slides)	Full sample (n=64)	ASCL2^{low} (n=20)	ASCL2^{high} (n=44)	p-value	Lgr5^{low} (n=8)	Lgr5^{high} (n=56)	p-value
Age				0.83			0.84
Mean (sd)	64.6 (11.4)	64.1 (12.7)	64.8 (10.8)		65.9 (9.6)	64.4 (11.7)	
Median (Min,Max)	64.5 (38,86)	64 (44,86)	65.5 (38,84)		69 (52,76)	64 (38,86)	
Gender				0.059			1
female	10 (16)	6 (30)	4 (9)		1 (12)	9 (16)	
male	54 (84)	14 (70)	40 (91)		7 (88)	47 (84)	
Stage				0.48			*0.0032*
Ⅰ/Ⅱ	13 (20)	6 (30)	7 (16)		1 (12)	12 (21)	
Ⅲ	34 (53)	9 (45)	25 (57)		1 (12)	33 (59)	
Ⅳ	17 (27)	5 (25)	12 (27)		6 (75)	11 (20)	
Neoadjuvant chemotherapy				0.56			0.42
No	44 (69)	12 (63)	32 (71)		7 (88)	37 (66)	
Yes	20 (31)	7 (37)	13 (29)		1 (12)	19 (34)	
Nodes positive				*0.027*			*0.039*
Mean (sd)	5.1 (7.2)	3.5 (5.4)	5.8 (7.8)		12.2 (14.5)	4.1 (4.9)	
Median (Min,Max)	3 (0,45)	1 (0,18)	4 (0,45)		8 (0,45)	3 (0,24)	
Nodes sampled				0.94			0.66
Mean (sd)	22.5 (10.9)	21.6 (11.5)	23 (10.7)		24.4 (13.7)	22.3 (10.6)	
Median (Min,Max)	21.5 (0,49)	22 (0,40)	21.5 (2,49)		26.5 (6,45)	21 (0,49)	
Histological grade				0.27			0.82
G1	3 (5)	1 (5)	2 (5)		0 (0)	3 (5)	
G2	37 (58)	9 (45)	28 (64)		4 (50)	33 (59)	
G3	24 (38)	10 (50)	14 (32)		4 (50)	20 (36)	

Table 5. Univariable and Multivariable analysis of overall survival (TMA)

Univariable

Covariate n=62 (46events)	HR (95%CI)	p-value	Global p-value
Age	1.01 (0.99,1.03)		0.44
Gender			0.076
female	Reference		
male	2.32 (0.92,5.9)		
Histological grade			0.3
G1	Reference		
G2	0.97 (0.29,3.24)	0.97	
G3	1.58 (0.46,5.44)	0.47	
Stage			0.001
Ⅰ/Ⅱ	Reference		
Ⅲ	2.43 (1.04,5.7)	0.041	
Ⅳ	5.62 (2.2,14.36)	<0.001	
Node positive	1.07 (1.03,1.11)		0.001
ASCL2 score			0.031
low	Reference		
high	2.07 (1.07,4.02)		
Lgr5 score			0.4
low	Reference		
high	0.7 (0.31,1.58)		

Multivariable

Covariate n=62 (46events)	HR (95%CI)	p-value	Global p-value
ASCL2 score			0.011
low	Reference		
high	2.47 (1.23,4.95)		
Gender			0.047
female	Reference		
male	2.61 (1.01,6.72)		
Stage			0.001
Ⅰ/Ⅱ	Reference		
Ⅲ	2.96 (1.23,7.13)	0.016	
Ⅳ	8.15 (2.99,22.2)	0.001	

Table 6. Univariable and multivariable analysis of progression-free survival (TMA)

| Univariable				Multivariable			
Covariate n=64 (50 events)	HR (95%CI)	p-value	Global p-value	Covariate n=64 (50 events)	HR (95%CI)	p-value	Global p-value
Age	1.00 (0.97,1.02)		0.84	ASCL2 score			*0.038*
				low	Reference		
Gender			*0.041*	high	2.05 (1.04,4.02)		
female	Reference			Gender			0.052
male	2.64 (1.04,6.7)			female	Reference		
Histological grade			0.61	male	2.54 (0.99,6.5)		
G1	Reference						
G2	0.88 (0.26,2.89)	0.83					
G3	1.18 (0.35,4.03)	0.79		Stage			*<0.001*
				Ⅰ/Ⅱ	Reference		
				Ⅲ	2.23 (0.99,5.04)	0.054	
Stage			*0.025*	Ⅳ	5.97 (2.31,15.41)		*<0.001*
Ⅰ/Ⅱ	Reference						
Ⅲ	2.05 (0.92,4.54)	0.078					
Ⅳ	4.62 (1.89,11.32)	*<0.001*					
Node positive	1.06 (1.03,1.09)		*<0.001*				
ASCL2 score			*0.05*				
low	Reference						
high	1.89 (1,3.57)						
Lgr5 score			0.62				
low	Reference						
high	0.82 (0.37,1.82)						

Figures
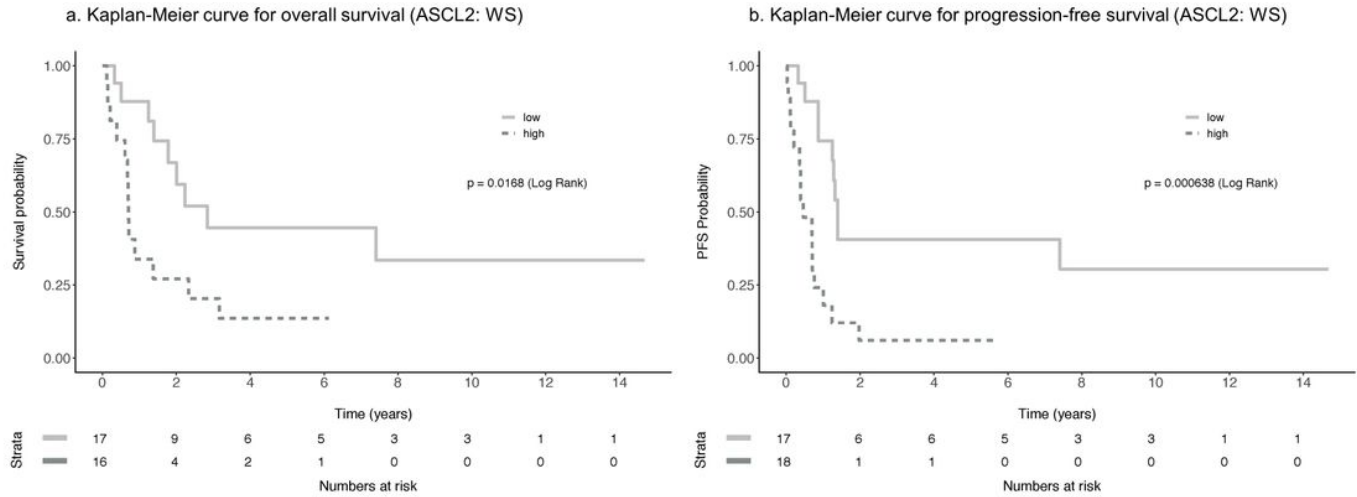
Figure 1

Survival analysis of 35 esophageal adenocarcinoma (EAC) whole slides (WS) by Kaplan-Meier (KM) curve. a. Overall survival rate (OS) in patients with high Ascl2 protein expression (dashed line) was significantly lower than that in patients with low Ascl2 expression (solid line), b. Progression-free survival (PFS) in patients with high Ascl2 protein expression (dashed line) was significantly lower than that in patients with low Ascl2 expression (solid line).
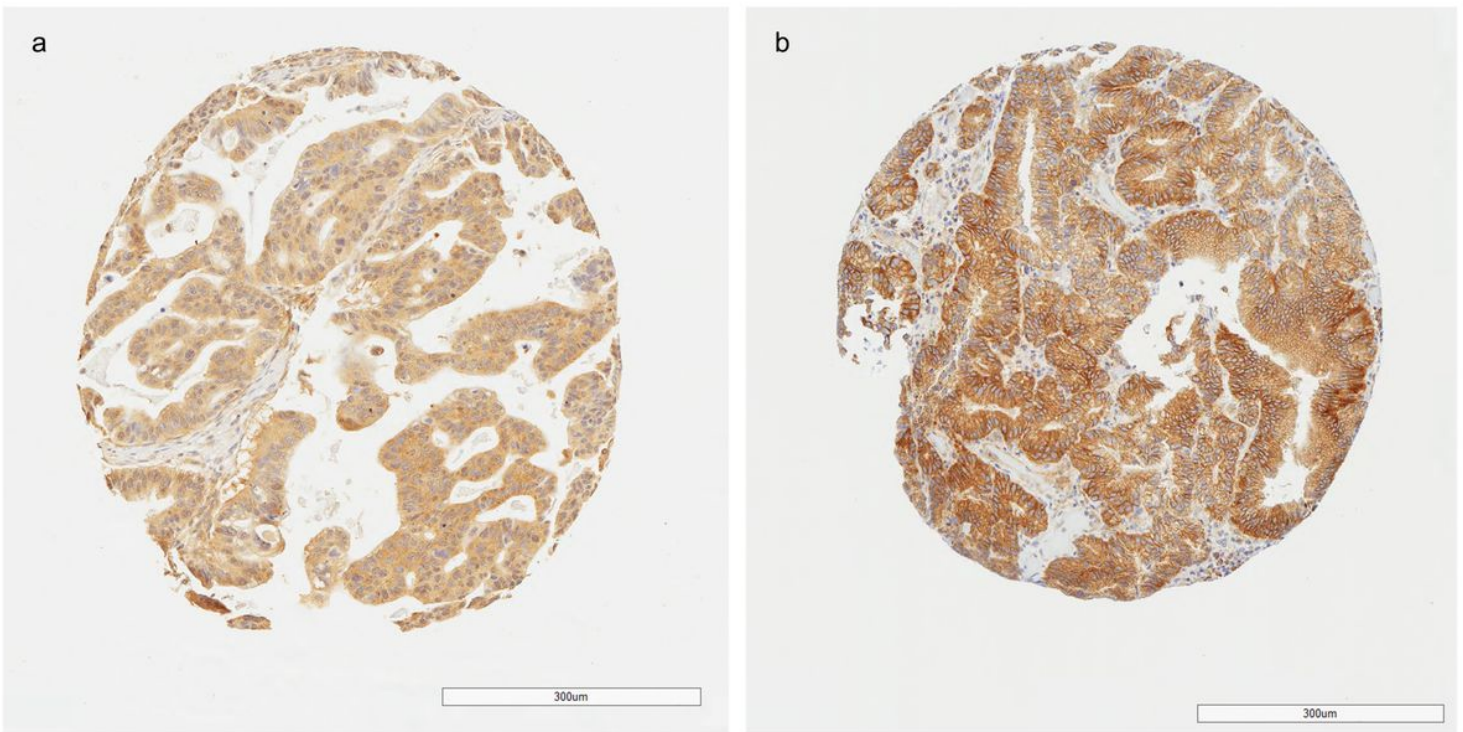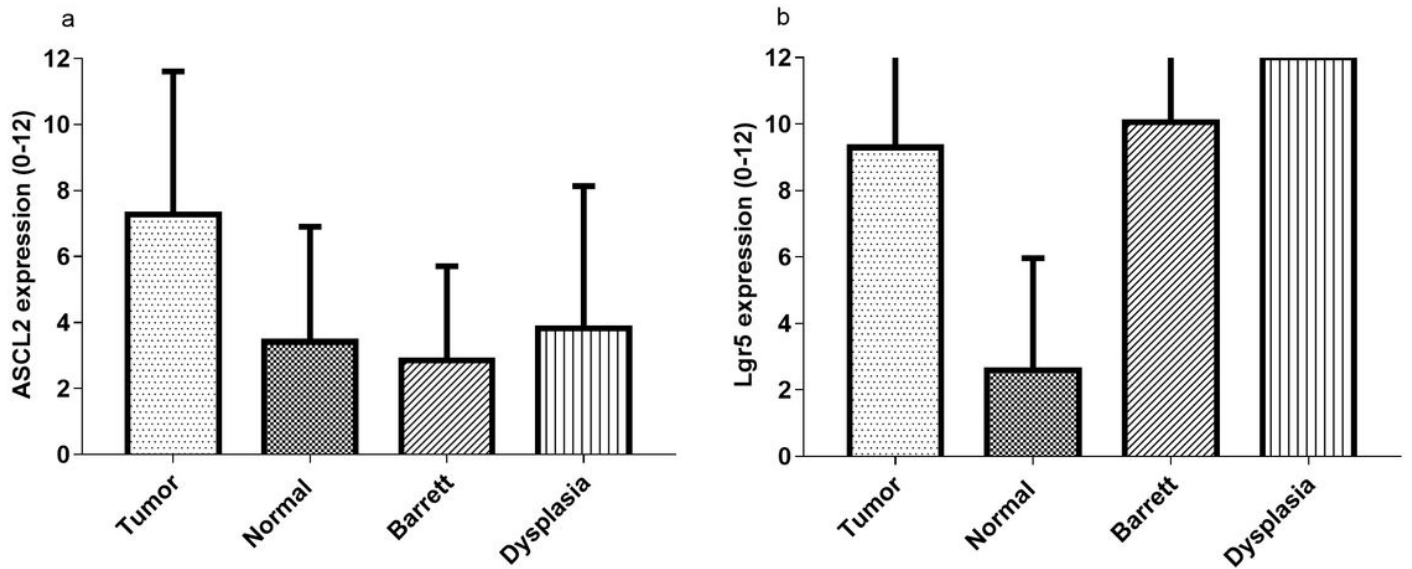
Figure 2

a. Ascl2 (cytoplasmic); b. Lgr5 (cytoplasmic); Immunohistochemical localization of protein in EAC tumor cell (original magnification x200).
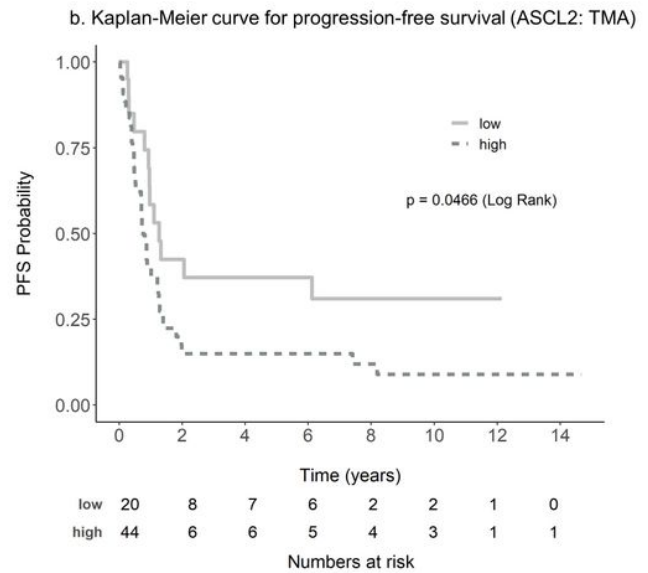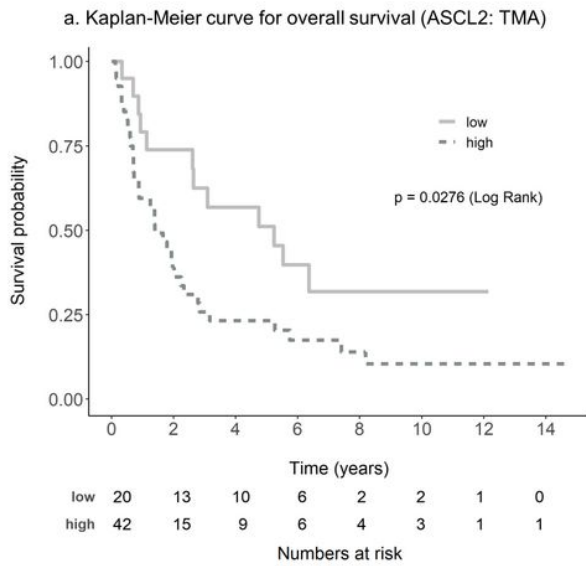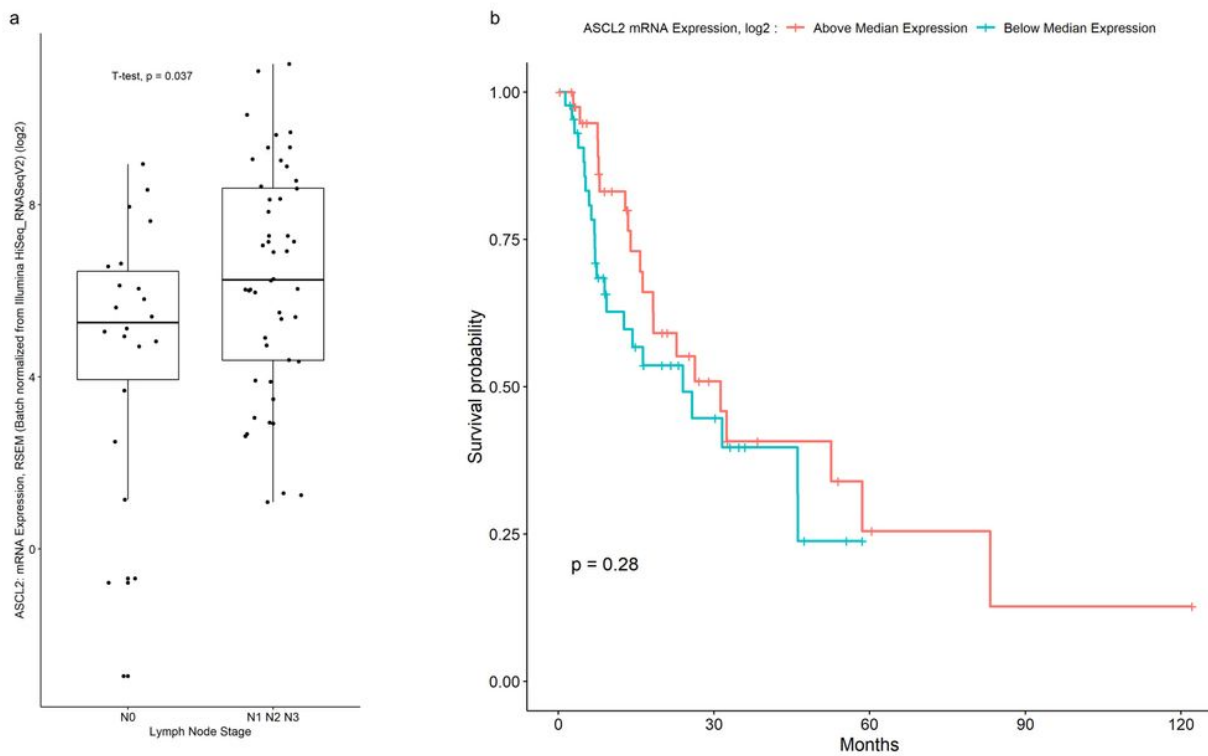

Figure 3

Survival analysis of 64 esophageal adenocarcinoma (EAC) patients by Kaplan-Meier (KM) curve. a. Overall survival rate (OS) in patients with high Ascl2 protein expression (dashed line) was significantly lower than that in patients with low Ascl2 expression (solid line), b. Progression-free survival (PFS) in patients with high Ascl2 protein expression (dashed line) was significantly lower than that in patients with low Ascl2 expression (solid line).

Figure 4

a. Ascl2; b. Lgr5; Immunohistochemical differential expression in EAC, Normal (squamous epithelium), Barrett's esophagus and dysplasia.

Figure 5

(A) Unpaired t-test shows patients with nodal status N0 have lower ASCL2 mRNA expression compared to patients with nodal status N1, N2, and N3. (B) Survival analysis of 86 EAC patients using the TCGA dataset by Kaplan-Meier (KM) curve.