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SULF2 expression by immunohistochemistry is a prognostic indicator in esophageal cancer: a cohort study

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ABSTRACT

Objectives Esophageal cancer is the eighth most commonly diagnosed cancer worldwide, and there is a need for biomarkers to improve diagnosis, prognosis, and treatment. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in patients with esophageal cancer.

Design Cohort study

Setting Single tertiary care center

Participants We included patients who underwent esophagectomy for invasive esophageal adenocarcinoma and squamous cell carcinoma at a tertiary care center from 1997 to 2006. We excluded patients with recurrent esophageal cancer or less than 3 mm invasive tumor on H&E stained slide. A section from each paraffin-embedded tissue specimen was stained with an anti-SULF2 monoclonal antibody.

Outcome measures A pathologist blinded to overall survival determined the percentage and intensity of tumor cells staining. Vital status was obtained through the Social Security Death Master File, and overall survival was calculated from the date of surgery.

Results One-hundred patients with invasive esophageal cancer were identified, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma. The squamous cell carcinoma samples had a higher mean percentage and intensity of tumor cells staining compared to the adenocarcinoma samples. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the hazard ratio for death increased by 13% (95% confidence interval, 1.01-1.25; p=0.03).

Conclusions The majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had SULF2 staining. The percentage of tumor cells staining for SULF2 was significantly associated with overall survival. Thus, SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

ARTICLE SUMMARY

Article focus

1. Esophageal cancer is the eighth most commonly diagnosed cancer and sixth most common cause of cancer death worldwide. There is a desperate need for biomarkers to improve diagnosis, prognosis, and treatment of this disease.
2. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis in several types of cancer.
3. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in 100 patients with esophageal cancer.

Key messages

1. We show for the first time SULF2 staining in esophageal cancer, including the majority of adenocarcinoma samples and all of the squamous cell carcinoma samples.
2. The percentage of tumor cells staining for SULF2 is significantly associated with overall survival in multivariate analysis.
3. SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

Strengths and Imitations of this study

1. A major strength of this study is the use tumor samples from a large, carefully selected cohort of patients with esophageal cancer.
2. Another major strength of the study is the novelty of SULF2, which may be a hub in the network of signaling pathways critical for cancer development and progression.
3. A limitation of this study is the lack of functional data that confirm causality of SULF2 in esophageal cancer cell lines. However, the significant association between increased SULF2 expression and worse overall survival in patients with esophageal cancer justifies investigation into the role of SULF2 in esophageal cancer cells, beyond the scope of the present study.
4. Another limitation of this study is the variability of SULF2 staining across samples. While SULF2 expression by immunohistochemistry is detected in the majority of the esophageal tumors, it is possible that SULF2 will be most useful as a biomarker in a subset of patients with esophageal cancer.

INTRODUCTION

Esophageal cancer is the eighth most commonly diagnosed cancer and the sixth most common cause of cancer death worldwide.[1] There are two main histologic types, each with distinct risk factors, geographic patterns, and temporal trends. Esophageal adenocarcinoma is associated with gastroesophageal reflux disease, obesity, and the precursor lesion Barrett's esophagus; its incidence has increased faster than that of any other cancer in the United States in the past few decades.[2] Esophageal squamous cell carcinoma is associated with tobacco smoking, alcohol consumption, and poor nutrition; its incidence remains much higher than that of esophageal adenocarcinoma in most of the world.[3] Patients with esophageal cancer continue to have a poor prognosis, with five-year overall survival still less than 15%.[4] A greater understanding of the molecular basis of esophageal cancer, including the development of new biomarkers, is greatly needed to improve the diagnosis, prognosis, and treatment of patients with this disease.

Heparan sulfate proteoglycans (HSPGs) consist of core proteins that are modified by the covalent addition of heparan sulfate chains.[5] These chains are composed of repeating disaccharide units, which are variably sulfated at four different positions. HSPGs perform enumerable signaling functions, using their sulfated chains to bind diverse protein ligands, such as growth factors, morphogens, and cytokines. These interactions depend on the pattern of the sulfation modifications with the 6-O-sulfation of glucosamine (6OS) known to be key for binding many ligands.[6] Two recently discovered sulfatases (SULF1 and SULF2) provide a novel mechanism for the regulation of HSPG-dependent signaling by acting on 6OS on the outside of cells. Work by us and others has shown that SULFs are neutral pH, extracellular enzymes which remove 6OS from intact HSPGs; they promote key signaling pathways by mobilizing protein ligands (e.g., Wnt ligands, GDNF, and BMP-4) from HSPG sequestration, thus liberating the ligands for binding to signal transduction receptors.[7-10]

One or both SULFs are broadly overexpressed at the transcript level in many human cancers, including non-small cell lung cancer, hepatocellular carcinoma, breast cancer, head and neck cancer, pancreatic adenocarcinoma, multiple myeloma, gastric carcinoma, and glioblastomas.[11,12] SULF2 has been directly implicated as a driver of carcinogenesis in pancreatic cancer[13], murine and human glioblastoma[14], hepatocellular carcinoma[15], and non-small cell lung cancer[16]. Moreover, *SULF2* promoter methylation and expression has been associated with overall survival in lung cancer and hepatocellular carcinoma, respectively.[15,17] However, there are no reports on SULF2 expression in esophageal cancer. This study evaluated SULF2 expression by immunohistochemistry and its association with overall survival in a cohort of patients with esophageal cancer.

METHODS

Patients

We identified patients who underwent esophagectomy at UCSF during the 10-year period from 1997 to 2006. We included patients undergoing primary resection for invasive esophageal adenocarcinoma and squamous cell carcinoma. Patients undergoing salvage surgery for recurrent esophageal cancer were excluded. We evaluated cases with at least 3 mm of invasive carcinoma on histologic sections and for which corresponding paraffin blocks were available. Clinical data was obtained through review of electronic medical records. Histologic data was obtained through review of pathology reports and confirmed by review of H&E stained sections by a pathologist. Pathologic stage was determined by the 7th edition of the American Joint Committee on Cancer (AJCC) staging system.[18] Vital status was obtained through the Social Security Death Master File. Overall survival was calculated from the date of surgery. The UCSF institutional review board approved this study.

Immunohistochemistry

A 5 μm section from each paraffin-embedded tissue specimen was stained with a mouse monoclonal antibody to SULF2 (2B4)[16] at a concentration of 2 μg/ml with avidin-biotin blocking. A pathologist (Annemieke van Zante) blinded to patient outcome determined the percentage and intensity of tumor cells staining. The percentage of tumor cells staining was scored from 0 to 100%. The intensity of tumor cells staining was assessed at 100x magnification and scored from 0 to 3. A score of 0 represented no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. When specimens showed a range of intensity, the mean intensity was recorded. The presence of endothelial cell staining was assessed for each slide and functioned as an internal positive control. Staining of tumor-associated stroma was also noted.

Statistical Analysis

Patient baseline characteristics and immunohistochemistry scores were summarized and compared by histologic type, using the Student's t-test for continuous variables and the chi-squared test for categorical variables. Survival analysis was performed using univariate and multivariate Cox proportional hazards models. Age, sex, and race were included in the multivariate model a priori. Histologic type, stage, grade, neoadjuvant therapy, and year of operation were included in the multivariate analysis only if the p-value was less than 0.10 in the univariate analysis. We repeated our analyses in pre-specified subgroups by histologic type (adenocarcinoma and squamous cell carcinoma) and neoadjuvant therapy (yes and no). In order to account for the possible misdiagnosis of gastric adenocarcinoma arising at the gastroesophageal junction as esophageal adenocarcinoma, we also repeated our analyses in the subgroup of patients with adenocarcinoma, excluding those with tumors located at the gastroesophageal junction and not associated with

Barrett's esophagus. For all statistical tests, a two-sided alpha level of 0.05 was considered statistically significant. Analyses were performed using Stata version 11.

RESULTS

Patients

We identified 233 patients who underwent esophagectomy at UCSF during the 10-year period from 1997 to 2006 (Figure 1). Of these, 100 patients met our inclusion criteria, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma.

There were several differences in baseline characteristics between patients with adenocarcinoma and those with squamous cell carcinoma (Table 1). Patients with adenocarcinoma had a higher proportion of men (82% vs. 52%; p<0.0005). Adenocarcinomas were more often located at the gastroesophageal junction and associated with Barrett's esophagus. Patients with adenocarcinoma had a higher frequency of neoadjuvant therapy and were generally diagnosed and resected at an earlier pathologic stage. There was no difference in age, race, or ethnicity between histologic types.

Table 1. Patient baseline characteristics.

	All patients (N=100)	Patients with AC (N=75)	Patients with SCC (N=25)	p-value†
Age, mean ± SD—years	64.2 ± 11.6	63.9 ± 11.0	65.2 ± 13.5	0.63
Sex—no. (%)				<0.0005
Female	21 (21)	9 (12)	12 (48)	
Male	79 (79)	66 (88)	13 (52)	
Race—no. (%)				0.13
White	80 (80)	62 (84)	18 (72)	
Asian	4 (4)	1 (1)	3 (12)	
Black	3 (3)	2 (3)	1 (4)	
Missing	13 (13)	10 (13)	3 (12)	

Ethnicity—no. (%)				0.48
Non-Hispanic	90 (90)	66 (88)	24 (96)	
Hispanic	2 (2)	2 (3)	0	
Missing	8 (8)	7 (9)	1 (4)	
Location of tumor‡—no. (%)				<0.0005
Upper esophagus	5 (5)	0	5 (20)	
Middle esophagus	9 (9)	2 (3)	7 (28)	
Lower esophagus	31 (31)	20 (27)	11 (44)	
Gastro-esophageal junction	55 (55)	53 (71)	2 (8)	
Presence of Barrett's esophagus—no. (%)				<0.0005
Yes	46 (46)	46 (61)	0	
No	54 (54)	29 (39)	25 (100)	
Pathologic stage—no. (%)				0.06
I	30 (30)	27 (36)	3 (12)	
II	31 (31)	23 (31)	8 (32)	
III	37 (37)	23 (31)	14 (56)	
IV	2 (2)	2 (3)	0	
Histologic grade—no. (%)				0.10
1 (Well-differentiated)	11	11	0	
2 (Moderately differentiated)	41	30	11	
3 (Poorly differentiated)	44	30	14	
4 (Undifferentiated)	4	4	0	
Neoadjuvant therapy				0.04
Yes	28 (28)	25 (33)	3 (12)	
No	72 (72)	50 (67)	22 (88)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; SD, standard deviation.

† P-values were calculated using the t-test for continuous variables and the chi-squared test for categorical variables.

‡ Location of tumor was recorded as the most superior location of the tumor in the esophagus. For example, if the pathology report noted tumor at the upper and middle esophagus, the location was recorded as upper esophagus.

Immunohistochemistry

SULF2 staining was detected on 93/100 of the specimens, including 68/75 (91%) adenocarcinoma samples and 25/25 (100%) squamous cell carcinoma samples (Figure 2). All samples had endothelial cell staining, which served as an internal positive control.

The squamous cell carcinoma samples had a higher mean percentage (49% vs. 36%; p=0.06) and intensity (1.9 vs. 1.4; p=0.0007) of tumor cells staining compared to the adenocarcinoma samples. Samples from patients who underwent neoadjuvant therapy had a lower mean intensity of tumor cells staining (1.3 vs. 1.6; p=0.03), but this difference was not evident when patients were stratified by histologic type. Neither percentage nor intensity of tumor cells staining was significantly associated with the other clinical and pathologic variables, including stage and grade.

SULF2 staining was present in some adjacent tissues. Among the 75 adenocarcinoma samples, incidental adjacent tissue included squamous epithelium (33), gastric epithelium (24), and Barrett's esophagus (20). Seventeen (52%) of the samples of adjacent benign squamous epithelium showed SULF2 staining of this epithelium. Staining was generally focal in the basal layer with weak to moderate intensity. Seven (29%) of the samples of adjacent benign gastric epithelium showed SULF2 staining of this epithelium, mostly patchy in the basal layer with weak to moderate intensity. Fifteen (75%) of the samples of Barrett's esophagus showed SULF2 staining of this metaplastic epithelium, mostly patchy with weak to moderate intensity (Figure 3).

Among the 25 squamous cell carcinoma cases, incidental adjacent tissue included benign squamous epithelium (6), dysplastic squamous epithelium (4), and carcinoma in situ (5). Three (50%) of the samples of adjacent benign squamous epithelium had SULF2 staining, mostly in the basal to middle layers with weak to moderate intensity. One (25%) of the samples of dysplastic squamous mucosa demonstrated SULF-2 staining. Interestingly, the area with low-grade dysplasia had weak intensity, and the area with high-grade dysplasia had moderate intensity. All 5 (100%) of the samples of carcinoma in situ had SULF2 staining, mostly in the basal layer with moderate intensity.

Survival analysis

Median follow-up time was 53.6 months (inter-quartile range, 15.0 to 97.6 months). Fifty-five patients died, including 38/75 (51%) patients with adenocarcinoma and 17/25 (68%) patients with squamous cell carcinoma.

In the univariate Cox proportional hazards models, pathologic stage, neoadjuvant therapy, and histologic type were significantly associated with overall survival; these were included in the multivariate model. Year of surgery and histologic grade were not significantly associated with survival; these were not included in the multivariate model. For every 10% increase in the percentage of tumor cells staining for SULF2, the risk of death increased by 4%, but this effect was not significant (p=0.42). Unadjusted Kaplan-Meier survival estimates confirmed these results (Figure 4).

In the multivariate Cox proportional hazards model, histologic type was no longer associated with overall survival (Table 2). Higher stage was still associated with worse survival (p=0.001), and patients who underwent neoadjuvant therapy still had a higher risk of death compared to those who did not (p=0.003). The percentage of tumor cells staining was significantly associated with overall survival. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increased by 13% (p=0.03).

Table 2. Univariate and multivariate Cox proportional hazards models for overall survival.

	Univariate Analysis			Multivariate Analysis		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
Age (per 10 years)	1.09	0.99-1.03	0.46	1.19	0.92-1.54	0.19
Sex						
Male	1			1		
Female	1.17	0.61-2.21	0.64	1.30	0.61-2.78	0.50
Race			0.59			0.40
White	1			1		
Asian	0.91	0.22-3.77	0.90	0.91	0.18-4.49	0.90
Black	2.21	0.53-9.17	0.27	3.76	0.80-17.57	0.09

Histologic type						
Adenocarcinoma	1			1		
Squamous cell	1.63	0.92-2.88	0.10	1.14	0.53-2.43	0.74
Pathologic stage			0.004			0.001
I	1			1		
II	2.42	1.08-5.38	0.03	2.65	1.13-6.22	0.03
III	4.01	1.88-8.55	<0.0005	5.14	2.23-11.82	<0.0005
IV	2.14	0.27-16.94	0.47	0.94	0.11-8.10	0.96
Neoadjuvant therapy						
No	1			1		
Yes	1.78	1.02-3.11	0.04	2.65	1.38-5.09	0.003
Percent (per 10%)	1.04	0.95-1.14	0.42	1.13	1.01-1.26	0.03

Only percentage, not intensity, of tumor cells staining was significantly associated with overall survival. Subgroup analysis by histologic type and neoadjuvant therapy did not show significant associations between percentage or intensity of tumor cells staining and survival.

DISCUSSION

This study showed that higher SULF2 expression by immunohistochemistry is associated with worse overall survival in esophageal cancer. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increases by 13%. As expected, higher stage was also significantly associated with worse overall survival. Interestingly, patients who underwent neoadjuvant therapy had worse overall survival than those who did not. This result likely reflects selection bias, in which patients diagnosed with more aggressive tumors are more likely to receive neoadjuvant therapy.

This study also showed that SULF2 staining differed by histologic type, likely reflecting their distinct etiologies. Squamous cell carcinoma samples had significantly higher percentage and intensity of tumor cells staining than adenocarcinoma samples. These results correspond to our previous findings in non-small cell lung cancer, in which in ten of ten squamous cell carcinoma samples showed staining for SULF-2, while zero out of ten adenocarcinoma samples

showed staining in the tumor cells.[16] However, in the aforementioned study, all squamous cell carcinoma and adenocarcinoma samples showed SULF2 staining of tumor stroma cells.

The association of SULF2 and overall survival corresponds to previous findings in two other types of cancer. Lai et al. showed that increased *SULF2* transcript expression was associated with worse overall survival in patients with hepatocellular carcinoma.[15] Tessema et al. showed that *SULF2* promoter methylation was associated with improved overall survival in patients with non-small cell lung cancer.[17] Our results show that increased SULF2 at the protein level is associated with worse overall survival in a third type of cancer, consistent with an important role for this extracellular enzyme in carcinogenesis.

The *SULFs* were first discovered in a study on quail embryo development, with the human orthologs, *SULF1* and *SULF2*, cloned soon thereafter.[10] SULFs have been shown to regulate several signaling pathways by changing the sulfation status of extracellular heparan sulfate proteoglycans. Ai et al. established that the SULFs promote canonical Wnt signaling, by removing 6OS from the heparan sulfate chains of heparan sulfate proteoglycans, allowing the Wnt ligands to interact with its Frizzled receptor, leading to activation of Wnt target genes.[7]

Originally, SULFs were thought to be tumor suppressors, because forced expression of a SULF in several tumor cell lines caused reduced growth-factor signaling by HB-EGF, FGF-2, or HGF, as well as diminished tumorigenicity.[19-22] Soon, however, they were thought to play an oncogenic role, as one or both SULF genes were found to be overexpressed in subsets of multiple tumors (breast, pancreatic, hepatocellular carcinoma, head and neck, lung, multiple myeloma, and glioblastoma).[10,11,13-16,20,23-25] SULF2 in particular has been identified as a candidate cancer-causing gene in two unbiased screening studies in human breast cancer and mouse brain cancer.[26,27] Moreover, in pancreatic and lung cancer cell lines, SULF2 knockdown led to reduced proliferation and reduced growth of xenografts in mice, likely due to its effect on canonical Wnt signaling pathway.[13,16] In hepatocellular cancer cell lines, overexpression of SULF2 led to increased proliferation and migration and markedly enhanced the tumorigenicity of the cells in nude mice.[15] Similarly, a recent study showed SULF2 transcripts and protein upregulation in human malignant astrocytoma, and, using knockdown and transgenic approaches, demonstrated a SULF2-dependent increase in PDGFR signaling, tumor cell proliferation in vitro, and tumor growth in vivo.[14] The present study is the first to investigate SULF2 in esophageal cancer.

Our results show that SULF2 was expressed in 93 of 100 human esophageal tumors. Importantly, high expression of SULF2 was associated with worse prognosis in esophageal cancer. As a secreted molecule that may

accumulate in blood or other body fluids, SULF-2 is a potential diagnostic or prognostic biomarker. Current screening programs for patients with Barrett's esophagus or severe gastroesophageal reflux are ineffective in preventing esophageal adenocarcinoma, due to the slow rate of progression and resultant low incidence of esophageal cancer.[4] The development of SULF2 as a biomarker may help identify a high-risk group that would make screening more feasible. In our study, 15 out of 20 (75%) adjacent Barrett's esophagus samples had SULF2 staining. Also, 5 out of 5 (100%) adjacent carcinoma in situ samples had SULF2 staining, although 3 out of 6 (50%) adjacent benign squamous epithelium had SULF2 staining as well. Future studies are needed to evaluate SULF2 specifically in these precursor lesions as well as in serum.

Given the prevalence and poor prognosis of esophageal cancer, advances in chemotherapy and targeted therapies based on key molecular pathways are greatly needed**.** Although further investigations are required for validation of the role of SULF2 in esophageal cancer, our results raise the possibility that SULF2 could be a therapeutic target. SULF2 is an extracellular enzyme and thus is potentially amenable to inhibition by either antibody-based or small-molecule drugs. *SULF1* and *SULF2* double knockout mice had increased perinatal mortality, and the mice that did survive had lower body weight.[28]; however *SULF1* and *SULF2* single knockout mice appear normal and have normal survival, suggesting that each enzyme might be singly targeted.

In conclusion, we show SULF2 expression in esophageal carcinoma. The vast majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had some degree of SULF2 protein expression. Higher percentage of tumor cells staining for SULF2 is significantly associated with worse overall survival in these patients. Patients with esophageal cancer have an extremely poor prognosis, and SULF2 is a promising biomarker that could play an important role in the diagnosis and prognosis.

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Competing Interests

None

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Contributorship Statement

NSL: study design, data collection, patient selection, statistical analysis and interpretation, paper writing; MVZ: patient selection, reviewing H&E slides, scoring SULF2 stained slides; SDR, DMJ: data analysis and interpretation; HLA: study design, data analysis and interpretation, paper writing.

Data Sharing Statement

No additional data available.

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Figure Legends

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.

Figure 2. Representative sections from adenocarcinoma samples with no staining **(A)**, weak staining **(B)**, and moderate staining **(C)**; and a squamous cell carcinoma sample with strong staining **(D)**. Endothelial cell staining served as an internal positive control, as seen in the upper left corner of **(A)**.

Figure 3. SULF2 staining of adjacent Barrett's esophagus.

Figure 4. Kaplan-Meier survival estimates by **(A)** stage, **(B)** histologic type, **(C)** neoadjuvant therapy, and **(D)** percentage of tumors cells staining. P-values were calculated using the log-rank test.

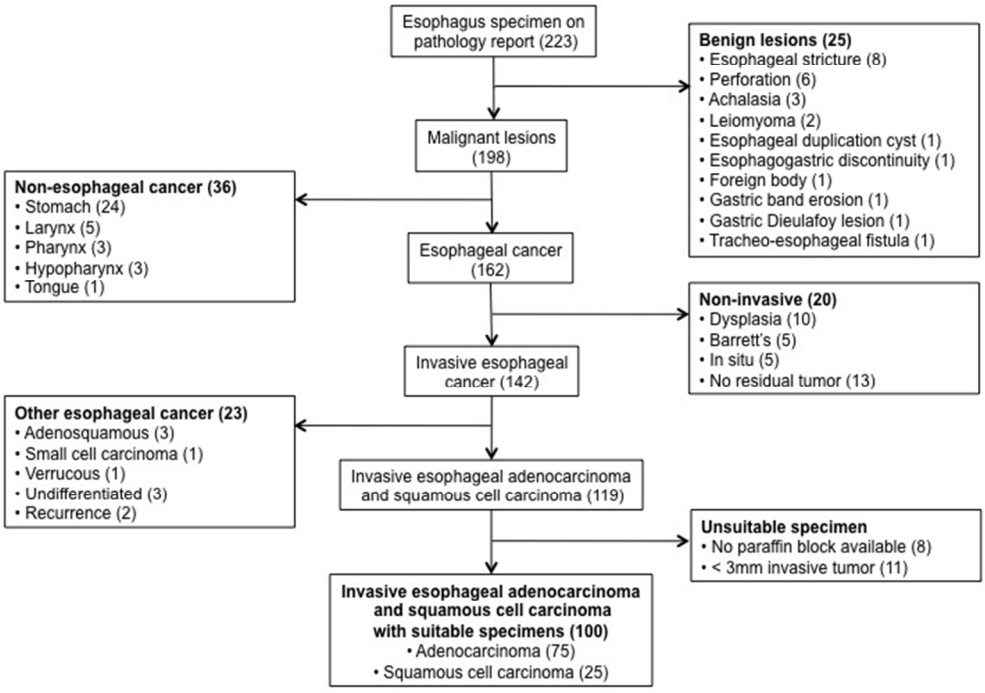

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.
254x190mm (72 x 72 DPI)

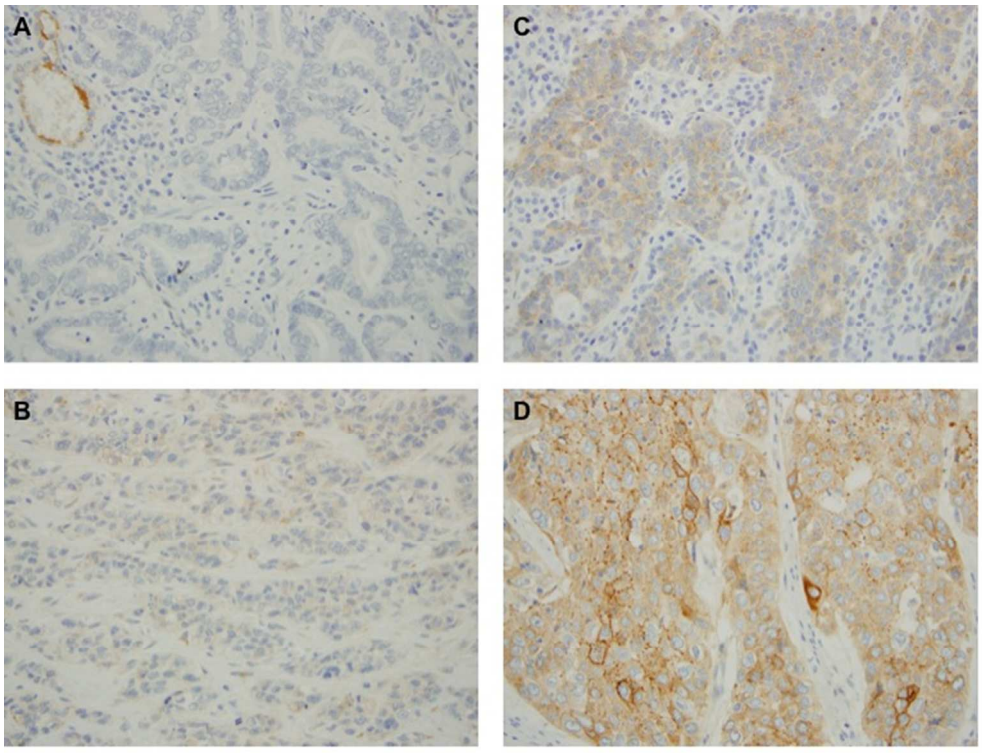

Figure 2. Representative sections from adenocarcinoma samples with no staining (A), weak staining (B), and moderate staining (C); and a squamous cell carcinoma sample with strong staining (D). Endothelial cell staining served as an internal positive control, as seen in the upper left corner of (A).
254x190mm (72 x 72 DPI)

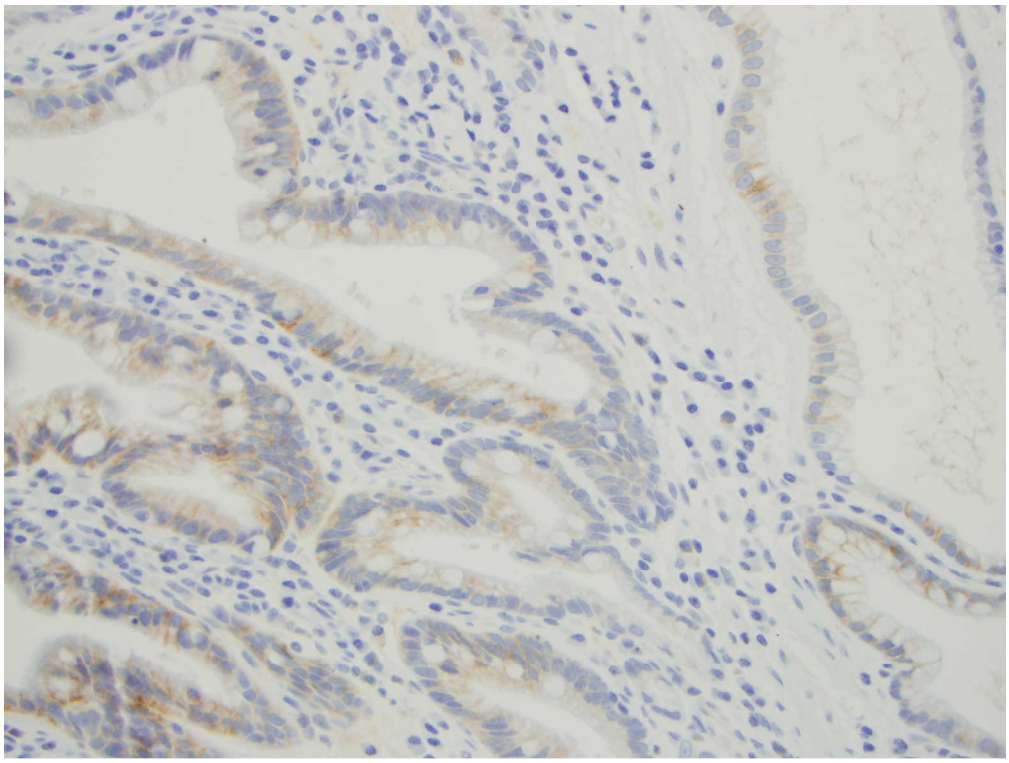

Figure 3. SULF2 staining of adjacent Barrett's esophagus.
239x180mm (216 x 216 DPI)

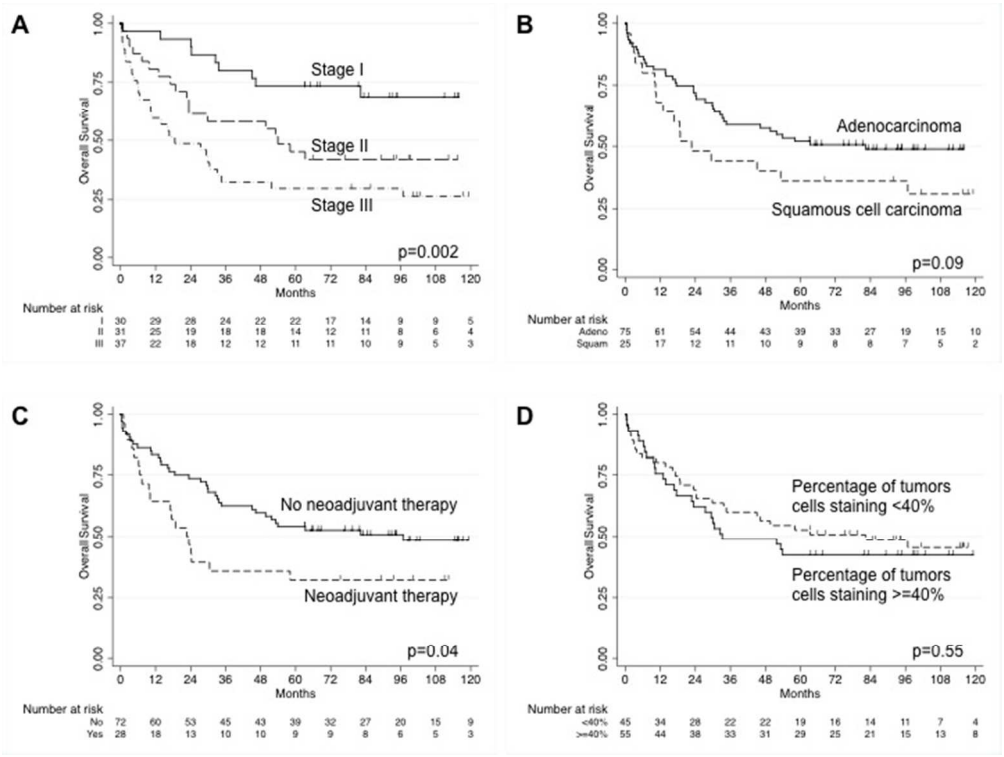

Figure 4. Kaplan-Meier survival estimates by (A) stage, (B) histologic type, (C) neoadjuvant therapy, and (D) percentage of tumors cells staining. P-values were calculated using the log-rank test.
254x190mm (72 x 72 DPI)

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SULF2 expression by immunohistochemistry and overall survival in esophageal cancer

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SULF2 expression by immunohistochemistry and overall survival in esophageal cancer: a cohort study

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ABSTRACT

Objectives Esophageal cancer is the eighth most commonly diagnosed cancer worldwide, and there is a need for biomarkers to improve diagnosis, prognosis, and treatment. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in patients with esophageal cancer.

Design Cohort study

Setting Single tertiary care center

Participants We included patients who underwent esophagectomy for invasive esophageal adenocarcinoma and squamous cell carcinoma at a tertiary care center from 1997 to 2006. We excluded patients with recurrent esophageal cancer or less than 3 mm invasive tumor on H&E stained slide. A section from each paraffin-embedded tissue specimen was stained with an anti-SULF2 monoclonal antibody.

Outcome measures A pathologist blinded to overall survival determined the percentage and intensity of tumor cells staining. Vital status was obtained through the Social Security Death Master File, and overall survival was calculated from the date of surgery.

Results One-hundred patients with invasive esophageal cancer were identified, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma. The squamous cell carcinoma samples had a higher mean percentage and intensity of tumor cells staining compared to the adenocarcinoma samples. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the hazard ratio for death increased by 13% (95% confidence interval, 1.01-1.25; p=0.03).

Conclusions The majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had SULF2 staining. The percentage of tumor cells staining for SULF2 was significantly associated with overall survival. Thus, SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

ARTICLE SUMMARY

Article focus

1. Esophageal cancer is the eighth most commonly diagnosed cancer and sixth most common cause of cancer death worldwide. There is a desperate need for biomarkers to improve diagnosis, prognosis, and treatment of this disease.
2. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis in several types of cancer.
3. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in 100 patients with esophageal cancer.

Key messages

1. We show for the first time SULF2 staining in esophageal cancer, including the majority of adenocarcinoma samples and all of the squamous cell carcinoma samples.
2. The percentage of tumor cells staining for SULF2 is significantly associated with overall survival in multivariate analysis.
3. SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

Strengths and Imitations of this study

1. A major strength of this study is the use tumor samples from a large, carefully selected cohort of patients with esophageal cancer.
2. Another major strength of the study is the novelty of SULF2, which may be a hub in the network of signaling pathways critical for cancer development and progression.
3. A limitation of this study is the lack of functional data that confirm causality of SULF2 in esophageal cancer cell lines. However, the significant association between increased SULF2 expression and worse overall survival in patients with esophageal cancer justifies investigation into the role of SULF2 in esophageal cancer cells, beyond the scope of the present study.
4. Another limitation of this study is the variability of SULF2 staining across samples. While SULF2 expression by immunohistochemistry is detected in the majority of the esophageal tumors, it is possible that SULF2 will be most useful as a biomarker in a subset of patients with esophageal cancer.

INTRODUCTION

Esophageal cancer is the eighth most commonly diagnosed cancer and the sixth most common cause of cancer death worldwide.[1] There are two main histologic types, each with distinct risk factors, geographic patterns, and temporal trends. Esophageal adenocarcinoma is associated with gastroesophageal reflux disease, obesity, and the precursor lesion Barrett's esophagus; its incidence has increased faster than that of any other cancer in the United States in the past few decades.[2] Esophageal squamous cell carcinoma is associated with tobacco smoking, alcohol consumption, and poor nutrition; its incidence remains much higher than that of esophageal adenocarcinoma in most of the world.[3] Patients with esophageal cancer continue to have a poor prognosis, with five-year overall survival still less than 15%.[4] A greater understanding of the molecular basis of esophageal cancer, including the development of new biomarkers, is greatly needed to improve the diagnosis, prognosis, and treatment of patients with this disease.

Heparan sulfate proteoglycans (HSPGs) consist of core proteins that are modified by the covalent addition of heparan or chondroitin sulfate chains.[5] These chains are composed of repeating disaccharide units, which are variably sulfated at four different positions. HSPGs perform enumerable signaling functions, using their sulfated chains to bind diverse protein ligands, such as growth factors, morphogens, and cytokines. These interactions depend on the pattern of the sulfation modifications with the 6-O-sulfation of glucosamine (6OS) known to be key for binding many ligands.[6] Two recently discovered sulfatases (SULF1 and SULF2) provide a novel mechanism for the regulation of HSPG-dependent signaling by acting on 6OS on the outside of cells. Work by us and others has shown that SULFs are neutral pH, extracellular enzymes which remove 6OS from intact HSPGs; they promote key signaling pathways by mobilizing protein ligands (e.g., Wnt ligands, GDNF, and BMP-4) from HSPG sequestration, thus liberating the ligands for binding to signal transduction receptors.[7-10]

One or both SULFs are broadly overexpressed at the transcript level in many human cancers, including non-small cell lung cancer, hepatocellular carcinoma, breast cancer, head and neck cancer, pancreatic adenocarcinoma, multiple myeloma, gastric carcinoma, and glioblastomas.[11,12] SULF2 has been directly implicated as a driver of carcinogenesis in pancreatic cancer[13], murine and human glioblastoma[14], hepatocellular carcinoma[15], and non-small cell lung cancer[16]. Moreover, *SULF2* promoter methylation and expression has been associated with overall survival in lung cancer and hepatocellular carcinoma, respectively.[15,17] However, there are no reports on SULF2 expression in esophageal cancer. This study evaluated SULF2 expression by immunohistochemistry and its association with overall survival in a cohort of patients with esophageal cancer.

METHODS

Patients

We identified patients who underwent esophagectomy at the University of California, San Francisco (UCSF) during the 10-year period from 1997 to 2006. We included patients undergoing primary resection for invasive esophageal adenocarcinoma and squamous cell carcinoma. Patients undergoing salvage surgery for recurrent esophageal cancer were excluded. We evaluated cases with at least 3 mm of invasive carcinoma on histologic sections and for which corresponding paraffin blocks were available. Clinical data was obtained through review of electronic medical records. Histologic data was obtained through review of pathology reports and confirmed by review of H&E stained sections by a pathologist. Pathologic stage was determined by the 7th edition of the American Joint Committee on Cancer (AJCC) staging system.[18] Vital status was obtained through the Social Security Death Master File. Overall survival was calculated from the date of surgery. The UCSF institutional review board approved this study.

Immunohistochemistry

A 5 μm section from each paraffin-embedded tissue specimen was stained with a mouse monoclonal antibody to SULF2 (AbD Serotec MCA5692T or Novus Biologicals NBP1-36727)[16] at a concentration of 2 μg/ml with avidin-biotin blocking. A pathologist blinded to patient outcome determined the percentage and intensity of tumor cells staining. The percentage of tumor cells staining was scored from 0 to 100%. The intensity of tumor cells staining was assessed at 100x magnification and scored from 0 to 3. A score of 0 represented no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. When specimens showed a range of intensity, the mean intensity was recorded. The presence of endothelial cell staining was assessed for each slide and functioned as an internal positive control. Staining of tumor-associated stroma was also noted.

Statistical Analysis

Patient baseline characteristics and immunohistochemistry scores were summarized and compared by histologic type, using the Student's t-test for continuous variables and the chi-squared test for categorical variables. Survival analysis was performed using univariate and multivariate Cox proportional hazards models. Age, sex, and race were included in the multivariate model a priori. Histologic type, stage, grade, neoadjuvant therapy, and year of operation were included in the multivariate analysis only if the p-value was less than 0.10 in the univariate analysis. We repeated our analyses in pre-specified subgroups by histologic type (adenocarcinoma and squamous cell carcinoma) and neoadjuvant therapy (yes and no). In order to account for the possible misdiagnosis of gastric adenocarcinoma arising at the gastroesophageal junction as esophageal adenocarcinoma, we also repeated our analyses in the subgroup of patients

with adenocarcinoma, excluding those with tumors located at the gastroesophageal junction and not associated with Barrett's esophagus. For all statistical tests, a two-sided alpha level of 0.05 was considered statistically significant. Analyses were performed using Stata version 11.

RESULTS

Patients

We identified 233 patients who underwent esophagectomy at UCSF during the 10-year period from 1997 to 2006 (Figure 1). Of these, 100 patients met our inclusion criteria, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma.

There were several differences in baseline characteristics between patients with adenocarcinoma and those with squamous cell carcinoma (Table 1). Patients with adenocarcinoma had a higher proportion of men (82% vs. 52%; p<0.0005). Adenocarcinomas were more often located at the gastroesophageal junction and associated with Barrett's esophagus. Patients with adenocarcinoma had a higher frequency of neoadjuvant therapy and were generally diagnosed and resected at an earlier pathologic stage. There was no difference in age, race, or ethnicity between histologic types.

Table 1. Patient baseline characteristics.

	All patients (N=100)	**Patients with AC (N=75)**	**Patients with SCC (N=25)**	**p-value†**
Age, mean ± SD—years	64.2 ± 11.6	63.9 ± 11.0	65.2 ± 13.5	0.63
Sex—no. (%)				<0.0005
Female	21 (21)	9 (12)	12 (48)	
Male	79 (79)	66 (88)	13 (52)	
Race—no. (%)	80 (80)	62 (84)	18 (72)	0.13
White	4 (4)	1 (1)	3 (12)	
Asian	3 (3)	2 (3)	1 (4)	
Black	13 (13)	10 (13)	3 (12)	

Missing				
Ethnicity—no. (%)				0.48
Non-Hispanic	90 (90)	66 (88)	24 (96)	
Hispanic	2 (2)	2 (3)	0	
Missing	8 (8)	7 (9)	1 (4)	
Location of tumor‡—no. (%)				<0.0005
Upper esophagus	5 (5)	0	5 (20)	
Middle esophagus	9 (9)	2 (3)	7 (28)	
Lower esophagus	31 (31)	20 (27)	11 (44)	
Gastro-esophageal junction	55 (55)	53 (71)	2 (8)	
Presence of Barrett's esophagus—no. (%)				<0.0005
Yes	46 (46)	46 (61)	0	
No	54 (54)	29 (39)	25 (100)	
Pathologic stage—no. (%)				0.06
I	30 (30)	27 (36)	3 (12)	
II	31 (31)	23 (31)	8 (32)	
III	37 (37)	23 (31)	14 (56)	
IV	2 (2)	2 (3)	0	
Histologic grade—no. (%)				0.10
1 (Well-differentiated)	11	11	0	
2 (Moderately differentiated)	41	30	11	
3 (Poorly differentiated)	44	30	14	
4 (Undifferentiated)	4	4	0	

Neoadjuvant therapy				0.04
Yes	28 (28)	25 (33)	3 (12)	
No	72 (72)	50 (67)	22 (88)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; SD, standard deviation.

† P-values were calculated using the t-test for continuous variables and the chi-squared test for categorical variables.

‡ Location of tumor was recorded as the most superior location of the tumor in the esophagus. For example, if the pathology report noted tumor at the upper and middle esophagus, the location was recorded as upper esophagus.

Immunohistochemistry

SULF2 staining was detected on 93/100 of the specimens, including 68/75 (91%) adenocarcinoma samples and 25/25 (100%) squamous cell carcinoma samples (Figure 2). All samples had endothelial cell staining, which served as an internal positive control.

The squamous cell carcinoma samples had a higher mean percentage (49% vs. 36%; p=0.06) and intensity (1.9 vs. 1.4; p=0.0007) of tumor cells staining compared to the adenocarcinoma samples. Samples from patients who underwent neoadjuvant therapy had a lower mean intensity of tumor cells staining (1.3 vs. 1.6; p=0.03), but this difference was not evident when patients were stratified by histologic type. Neither percentage nor intensity of tumor cells staining was significantly associated with the other clinical and pathologic variables, including stage and grade.

SULF2 staining was present in some adjacent tissues. Among the 75 adenocarcinoma samples, incidental adjacent tissue included squamous epithelium (33), gastric epithelium (24), and Barrett's esophagus (20). Seventeen (52%) of the samples of adjacent benign squamous epithelium showed SULF2 staining of this epithelium. Staining was generally focal in the basal layer with weak to moderate intensity. Seven (29%) of the samples of adjacent benign gastric epithelium showed SULF2 staining of this epithelium, mostly patchy in the basal layer with weak to moderate intensity. Fifteen (75%) of the samples of Barrett's esophagus showed SULF2 staining of this metaplastic epithelium, mostly patchy with weak to moderate intensity (Figure 3).

Among the 25 squamous cell carcinoma cases, incidental adjacent tissue included benign squamous epithelium (6), dysplastic squamous epithelium (4), and carcinoma in situ (5). Three (50%) of the samples of adjacent benign squamous epithelium had SULF2 staining, mostly in the basal to middle layers with weak to moderate intensity. One (25%) of the samples of dysplastic squamous mucosa demonstrated SULF2 staining. Interestingly, the area with low-

grade dysplasia had weak intensity, and the area with high-grade dysplasia had moderate intensity. All 5 (100%) of the samples of carcinoma in situ had SULF2 staining, mostly in the basal layer with moderate intensity.

Survival analysis

Median follow-up time was 53.6 months (inter-quartile range, 15.0 to 97.6 months). Fifty-five patients died, including 38/75 (51%) patients with adenocarcinoma and 17/25 (68%) patients with squamous cell carcinoma.

In the univariate Cox proportional hazards models, pathologic stage, neoadjuvant therapy, and histologic type were significantly associated with overall survival; these were included in the multivariate model. Year of surgery and histologic grade were not significantly associated with survival; these were not included in the multivariate model. For every 10% increase in the percentage of tumor cells staining for SULF2, the risk of death increased by 4%, but this effect was not significant (p=0.42). Unadjusted Kaplan-Meier survival estimates confirmed these results (Figure 4).

In the multivariate Cox proportional hazards model, histologic type was no longer associated with overall survival (Table 2). Higher stage was still associated with worse survival (p=0.001), and patients who underwent neoadjuvant therapy still had a higher risk of death compared to those who did not (p=0.003). The percentage of tumor cells staining was significantly associated with overall survival. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increased by 13% (p=0.03).

Table 2. Univariate and multivariate Cox proportional hazards models for overall survival.

	Univariate Analysis			**Multivariate Analysis**		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
Age (per 10 years)	1.09	0.99-1.03	0.46	1.19	0.92-1.54	0.19
Sex						
Male	1			1		
Female	1.17	0.61-2.21	0.64	1.30	0.61-2.78	0.50
Race	1	0.22-3.77	0.59	1	0.18-4.49	0.40

White	0.91	0.53-9.17		0.91	0.80-17.57	
Asian	2.21		0.90	3.76		0.90
Black			0.27			0.09
Histologic type						
Adenocarcinoma	1			1		
Squamous cell	1.63	0.92-2.88	0.10	1.14	0.53-2.43	0.74
Pathologic stage			0.004			0.001
I	1			1		
II	2.42	1.08-5.38	0.03	2.65	1.13-6.22	0.03
III	4.01	1.88-8.55	<0.0005	5.14	2.23-11.82	<0.0005
IV	2.14	0.27-16.94	0.47	0.94	0.11-8.10	0.96
Neoadjuvant therapy						
No	1			1		
Yes	1.78	1.02-3.11	0.04	2.65	1.38-5.09	0.003
Percent (per 10%)	1.04	0.95-1.14	0.42	1.13	1.01-1.26	0.03

Only percentage, not intensity, of tumor cells staining was significantly associated with overall survival. Subgroup analysis by histologic type and neoadjuvant therapy did not show significant associations between percentage or intensity of tumor cells staining and survival.

DISCUSSION

This study showed that higher SULF2 expression by immunohistochemistry is associated with worse overall survival in esophageal cancer. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increases by 13%. As expected, higher stage was also significantly associated with worse overall survival. Interestingly, patients who underwent neoadjuvant therapy had worse overall survival than those who did not. This result likely reflects selection bias, in which patients diagnosed with more aggressive tumors are more likely to receive neoadjuvant therapy.

This study also showed that SULF2 staining differed by histologic type, likely reflecting their distinct etiologies. Squamous cell carcinoma samples had significantly higher percentage and intensity of tumor cells staining than adenocarcinoma samples. These results correspond to our previous findings in non-small cell lung cancer, in which in ten of ten squamous cell carcinoma samples showed staining for SULF2, while zero out of ten adenocarcinoma samples showed staining in the tumor cells.[16] However, in the aforementioned study, all squamous cell carcinoma and adenocarcinoma samples showed SULF2 staining of tumor stroma cells.

The association of SULF2 and overall survival corresponds to previous findings in two other types of cancer. Lai et al. showed that increased *SULF2* transcript expression was associated with worse overall survival in patients with hepatocellular carcinoma.[15] Tessema et al. showed that *SULF2* promoter methylation was associated with improved overall survival in patients with non-small cell lung cancer.[17] Our results show that increased SULF2 at the protein level is associated with worse overall survival in a third type of cancer, consistent with an important role for this extracellular enzyme in carcinogenesis.

The *SULFs* were first discovered in a study on quail embryo development, with the human orthologs, *SULF1* and *SULF2*, cloned soon thereafter.[10] SULFs have been shown to regulate several signaling pathways by changing the sulfation status of extracellular heparan sulfate proteoglycans. Ai et al. established that the SULFs promote canonical Wnt signaling, by removing 6OS from the heparan sulfate chains of heparan sulfate proteoglycans, allowing the Wnt ligands to interact with its Frizzled receptor, leading to activation of Wnt target genes.[7]

Originally, SULFs were thought to be tumor suppressors, because forced expression of a SULF in several tumor cell lines caused reduced growth-factor signaling by HB-EGF, FGF-2, or HGF, as well as diminished tumorigenicity.[19-22] Soon, however, they were thought to play an oncogenic role, as one or both SULF genes were found to be overexpressed in subsets of multiple tumors (breast, pancreatic, hepatocellular carcinoma, head and neck, lung, multiple myeloma, and glioblastoma).[10,11,13-16,20,23-25] SULF2 in particular has been identified as a candidate cancer-causing gene in two unbiased screening studies in human breast cancer and mouse brain cancer.[26,27] Moreover, in pancreatic and lung cancer cell lines, SULF2 knockdown led to reduced proliferation and reduced growth of xenografts in mice, likely due to its effect on canonical Wnt signaling pathway.[13,16] In hepatocellular cancer cell lines, overexpression of SULF2 led to increased proliferation and migration and markedly enhanced the tumorigenicity of the cells in nude mice.[15] Similarly, a recent study showed SULF2 transcripts and protein upregulation in human malignant astrocytoma, and, using knockdown and transgenic approaches, demonstrated a SULF2-dependent increase in PDGFR signaling,

tumor cell proliferation in vitro, and tumor growth in vivo.[14] The present study is the first to investigate SULF2 in esophageal cancer.

Our results show that SULF2 was expressed in 93 of 100 human esophageal tumors. Importantly, high expression of SULF2 was associated with worse prognosis in esophageal cancer. As a secreted molecule, SULF2 is a potential diagnostic or prognostic biomarker. Current screening programs for patients with Barrett's esophagus or severe gastroesophageal reflux are ineffective in preventing esophageal adenocarcinoma, due to the slow rate of progression and resultant low incidence of esophageal cancer.[4] The development of SULF2 as a biomarker may help identify a high-risk group that would make screening more feasible. In our study, 15 out of 20 (75%) adjacent Barrett's esophagus samples had SULF2 staining. Also, 5 out of 5 (100%) adjacent carcinoma in situ samples had SULF2 staining, although 3 out of 6 (50%) adjacent benign squamous epithelium had SULF2 staining as well. Future studies are needed to evaluate SULF2 specifically in these precursor lesions as well as in blood or other body fluids.

Given the prevalence and poor prognosis of esophageal cancer, advances in chemotherapy and targeted therapies based on key molecular pathways are greatly needed**.** Although further investigations are required for validation of the role of SULF2 in esophageal cancer, our results raise the possibility that SULF2 could be a therapeutic target. SULF2 is an extracellular enzyme and thus is potentially amenable to inhibition by either antibody-based or small-molecule drugs. *SULF1* and *SULF2* double knockout mice had increased perinatal mortality, and the mice that did survive had lower body weight.[28]; however *SULF1* and *SULF2* single knockout mice appear normal and have normal survival, suggesting that each enzyme might be singly targeted.

In conclusion, we show SULF2 expression in esophageal carcinoma. The vast majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had some degree of SULF2 protein expression. Higher percentage of tumor cells staining for SULF2 is significantly associated with worse overall survival in these patients. Patients with esophageal cancer have an extremely poor prognosis, and SULF2 is a promising biomarker that could play an important role in the diagnosis and prognosis.

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Competing Interests

None

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Contributorship Statement

NSL: study design, data collection, patient selection, statistical analysis and interpretation, paper writing; MVZ: patient selection, reviewing H&E slides, scoring SULF2 stained slides; SDR, DMJ: data analysis and interpretation; HLA: study design, data analysis and interpretation, paper writing.

Data Sharing Statement

No additional data available.

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Figure Legends

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.

Figure 2. Representative sections from adenocarcinoma samples with no staining **(A)**, weak staining **(B)**, and moderate staining **(C)**; and a squamous cell carcinoma sample with strong staining **(D)**. Endothelial cell staining served as an internal positive control, as seen in the upper left corner of **(A)**.

Figure 3. SULF2 staining of adjacent Barrett's esophagus.

Figure 4. Kaplan-Meier survival estimates by **(A)** stage, **(B)** histologic type, **(C)** neoadjuvant therapy, and **(D)** percentage of tumors cells staining. P-values were calculated using the log-rank test.

SULF2 expression by immunohistochemistry ~~is a prognostic indicator~~and overall survival in esophageal cancer: a cohort study

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ABSTRACT

Objectives Esophageal cancer is the eighth most commonly diagnosed cancer worldwide, and there is a need for biomarkers to improve diagnosis, prognosis, and treatment. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in patients with esophageal cancer.

Design Cohort study

Setting Single tertiary care center

Participants We included patients who underwent esophagectomy for invasive esophageal adenocarcinoma and squamous cell carcinoma at a tertiary care center from 1997 to 2006. We excluded patients with recurrent esophageal cancer or less than 3 mm invasive tumor on H&E stained slide. A section from each paraffin-embedded tissue specimen was stained with an anti-SULF2 monoclonal antibody.

Outcome measures A pathologist blinded to overall survival determined the percentage and intensity of tumor cells staining. Vital status was obtained through the Social Security Death Master File, and overall survival was calculated from the date of surgery.

Results One-hundred patients with invasive esophageal cancer were identified, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma. The squamous cell carcinoma samples had a higher mean percentage and intensity of tumor cells staining compared to the adenocarcinoma samples. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the hazard ratio for death increased by 13% (95% confidence interval, 1.01-1.25; p=0.03).

Conclusions The majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had SULF2 staining. The percentage of tumor cells staining for SULF2 was significantly associated with overall survival. Thus, SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

ARTICLE SUMMARY

Article focus

1. Esophageal cancer is the eighth most commonly diagnosed cancer and sixth most common cause of cancer death worldwide. There is a desperate need for biomarkers to improve diagnosis, prognosis, and treatment of this disease.
2. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis in several types of cancer.
3. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in 100 patients with esophageal cancer.

Key messages

1. We show for the first time SULF2 staining in esophageal cancer, including the majority of adenocarcinoma samples and all of the squamous cell carcinoma samples.
2. The percentage of tumor cells staining for SULF2 is significantly associated with overall survival in multivariate analysis.
3. SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

Strengths and Imitations of this study

1. A major strength of this study is the use tumor samples from a large, carefully selected cohort of patients with esophageal cancer.
2. Another major strength of the study is the novelty of SULF2, which may be a hub in the network of signaling pathways critical for cancer development and progression.
3. A limitation of this study is the lack of functional data that confirm causality of SULF2 in esophageal cancer cell lines. However, the significant association between increased SULF2 expression and worse overall survival in patients with esophageal cancer justifies investigation into the role of SULF2 in esophageal cancer cells, beyond the scope of the present study.
4. Another limitation of this study is the variability of SULF2 staining across samples. While SULF2 expression by immunohistochemistry is detected in the majority of the esophageal tumors, it is possible that SULF2 will be most useful as a biomarker in a subset of patients with esophageal cancer.

INTRODUCTION

Esophageal cancer is the eighth most commonly diagnosed cancer and the sixth most common cause of cancer death worldwide.[1] There are two main histologic types, each with distinct risk factors, geographic patterns, and temporal trends. Esophageal adenocarcinoma is associated with gastroesophageal reflux disease, obesity, and the precursor lesion Barrett's esophagus; its incidence has increased faster than that of any other cancer in the United States in the past few decades.[2] Esophageal squamous cell carcinoma is associated with tobacco smoking, alcohol consumption, and poor nutrition; its incidence remains much higher than that of esophageal adenocarcinoma in most of the world.[3] Patients with esophageal cancer continue to have a poor prognosis, with five-year overall survival still less than 15%.[4] A greater understanding of the molecular basis of esophageal cancer, including the development of new biomarkers, is greatly needed to improve the diagnosis, prognosis, and treatment of patients with this disease.

Heparan sulfate proteoglycans (HSPGs) consist of core proteins that are modified by the covalent addition of heparan or chondroitin sulfate chains.[5] These chains are composed of repeating disaccharide units, which are variably sulfated at four different positions. HSPGs perform enumerable signaling functions, using their sulfated chains to bind diverse protein ligands, such as growth factors, morphogens, and cytokines. These interactions depend on the pattern of the sulfation modifications with the 6-O-sulfation of glucosamine (6OS) known to be key for binding many ligands.[6] Two recently discovered sulfatases (SULF1 and SULF2) provide a novel mechanism for the regulation of HSPG-dependent signaling by acting on 6OS on the outside of cells. Work by us and others has shown that SULFs are neutral pH, extracellular enzymes which remove 6OS from intact HSPGs; they promote key signaling pathways by mobilizing protein ligands (e.g., Wnt ligands, GDNF, and BMP-4) from HSPG sequestration, thus liberating the ligands for binding to signal transduction receptors.[7-10]

One or both SULFs are broadly overexpressed at the transcript level in many human cancers, including non-small cell lung cancer, hepatocellular carcinoma, breast cancer, head and neck cancer, pancreatic adenocarcinoma, multiple myeloma, gastric carcinoma, and glioblastomas.[11,12] SULF2 has been directly implicated as a driver of carcinogenesis in pancreatic cancer[13], murine and human glioblastoma[14], hepatocellular carcinoma[15], and non-small cell lung cancer[16]. Moreover, *SULF2* promoter methylation and expression has been associated with overall survival in lung cancer and hepatocellular carcinoma, respectively.[15,17] However, there are no reports on SULF2 expression in esophageal cancer. This study evaluated SULF2 expression by immunohistochemistry and its association with overall survival in a cohort of patients with esophageal cancer.

METHODS

Patients

We identified patients who underwent esophagectomy at the University of California, San Francisco (UCSF) during the 10-year period from 1997 to 2006. We included patients undergoing primary resection for invasive esophageal adenocarcinoma and squamous cell carcinoma. Patients undergoing salvage surgery for recurrent esophageal cancer were excluded. We evaluated cases with at least 3 mm of invasive carcinoma on histologic sections and for which corresponding paraffin blocks were available. Clinical data was obtained through review of electronic medical records. Histologic data was obtained through review of pathology reports and confirmed by review of H&E stained sections by a pathologist. Pathologic stage was determined by the 7th edition of the American Joint Committee on Cancer (AJCC) staging system.[18] Vital status was obtained through the Social Security Death Master File. Overall survival was calculated from the date of surgery. The UCSF institutional review board approved this study.

Immunohistochemistry

A 5 μm section from each paraffin-embedded tissue specimen was stained with a mouse monoclonal antibody to SULF2 (AbD Serotec MCA5692T or Novus Biologicals NBP1-36727)~~2B4)~~[16] at a concentration of 2 μg/ml with avidin-biotin blocking. A pathologist ~~(Annemieke van Zante)~~ blinded to patient outcome determined the percentage and intensity of tumor cells staining. The percentage of tumor cells staining was scored from 0 to 100%. The intensity of tumor cells staining was assessed at 100x magnification and scored from 0 to 3. A score of 0 represented no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. When specimens showed a range of intensity, the mean intensity was recorded. The presence of endothelial cell staining was assessed for each slide and functioned as an internal positive control. Staining of tumor-associated stroma was also noted.

Statistical Analysis

Patient baseline characteristics and immunohistochemistry scores were summarized and compared by histologic type, using the Student's t-test for continuous variables and the chi-squared test for categorical variables. Survival analysis was performed using univariate and multivariate Cox proportional hazards models. Age, sex, and race were included in the multivariate model a priori. Histologic type, stage, grade, neoadjuvant therapy, and year of operation were included in the multivariate analysis only if the p-value was less than 0.10 in the univariate analysis. We repeated our analyses in pre-specified subgroups by histologic type (adenocarcinoma and squamous cell carcinoma) and neoadjuvant therapy (yes and no). In order to account for the possible misdiagnosis of gastric adenocarcinoma arising at the gastroesophageal junction as esophageal adenocarcinoma, we also repeated our analyses in the subgroup of patients

with adenocarcinoma, excluding those with tumors located at the gastroesophageal junction and not associated with Barrett's esophagus. For all statistical tests, a two-sided alpha level of 0.05 was considered statistically significant. Analyses were performed using Stata version 11.

RESULTS

Patients

We identified 233 patients who underwent esophagectomy at UCSF during the 10-year period from 1997 to 2006 (Figure 1). Of these, 100 patients met our inclusion criteria, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma.

There were several differences in baseline characteristics between patients with adenocarcinoma and those with squamous cell carcinoma (Table 1). Patients with adenocarcinoma had a higher proportion of men (82% vs. 52%; p<0.0005). Adenocarcinomas were more often located at the gastroesophageal junction and associated with Barrett's esophagus. Patients with adenocarcinoma had a higher frequency of neoadjuvant therapy and were generally diagnosed and resected at an earlier pathologic stage. There was no difference in age, race, or ethnicity between histologic types.

Table 1. Patient baseline characteristics.

	All patients (N=100)	Patients with AC (N=75)	Patients with SCC (N=25)	p-value†
Age, mean ± SD—years	64.2 ± 11.6	63.9 ± 11.0	65.2 ± 13.5	0.63
Sex—no. (%)				<0.0005
Female	21 (21)	9 (12)	12 (48)	
Male	79 (79)	66 (88)	13 (52)	
Race—no. (%)	80 (80)	62 (84)	18 (72)	0.13
White	4 (4)	1 (1)	3 (12)	
Asian	3 (3)	2 (3)	1 (4)	
Black	13 (13)	10 (13)	3 (12)	

Missing				
Ethnicity—no. (%)				0.48
Non-Hispanic	90 (90)	66 (88)	24 (96)	
Hispanic	2 (2)	2 (3)	0	
Missing	8 (8)	7 (9)	1 (4)	
Location of tumor‡—no. (%)				<0.0005
Upper esophagus	5 (5)	0	5 (20)	
Middle esophagus	9 (9)	2 (3)	7 (28)	
Lower esophagus	31 (31)	20 (27)	11 (44)	
Gastro-esophageal junction	55 (55)	53 (71)	2 (8)	
Presence of Barrett's esophagus—no. (%)				<0.0005
Yes	46 (46)	46 (61)	0	
No	54 (54)	29 (39)	25 (100)	
Pathologic stage—no. (%)				0.06
I	30 (30)	27 (36)	3 (12)	
II	31 (31)	23 (31)	8 (32)	
III	37 (37)	23 (31)	14 (56)	
IV	2 (2)	2 (3)	0	
Histologic grade—no. (%)				0.10
1 (Well-differentiated)	11	11	0	
2 (Moderately differentiated)	41	30	11	
3 (Poorly differentiated)	44	30	14	
4 (Undifferentiated)	4	4	0	

Neoadjuvant therapy				0.04
Yes	28 (28)	25 (33)	3 (12)	
No	72 (72)	50 (67)	22 (88)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; SD, standard deviation.

† P-values were calculated using the t-test for continuous variables and the chi-squared test for categorical variables.

‡ Location of tumor was recorded as the most superior location of the tumor in the esophagus. For example, if the pathology report noted tumor at the upper and middle esophagus, the location was recorded as upper esophagus.

Immunohistochemistry

SULF2 staining was detected on 93/100 of the specimens, including 68/75 (91%) adenocarcinoma samples and 25/25 (100%) squamous cell carcinoma samples (Figure 2). All samples had endothelial cell staining, which served as an internal positive control.

The squamous cell carcinoma samples had a higher mean percentage (49% vs. 36%; p=0.06) and intensity (1.9 vs. 1.4; p=0.0007) of tumor cells staining compared to the adenocarcinoma samples. Samples from patients who underwent neoadjuvant therapy had a lower mean intensity of tumor cells staining (1.3 vs. 1.6; p=0.03), but this difference was not evident when patients were stratified by histologic type. Neither percentage nor intensity of tumor cells staining was significantly associated with the other clinical and pathologic variables, including stage and grade.

SULF2 staining was present in some adjacent tissues. Among the 75 adenocarcinoma samples, incidental adjacent tissue included squamous epithelium (33), gastric epithelium (24), and Barrett's esophagus (20). Seventeen (52%) of the samples of adjacent benign squamous epithelium showed SULF2 staining of this epithelium. Staining was generally focal in the basal layer with weak to moderate intensity. Seven (29%) of the samples of adjacent benign gastric epithelium showed SULF2 staining of this epithelium, mostly patchy in the basal layer with weak to moderate intensity. Fifteen (75%) of the samples of Barrett's esophagus showed SULF2 staining of this metaplastic epithelium, mostly patchy with weak to moderate intensity (Figure 3).

Among the 25 squamous cell carcinoma cases, incidental adjacent tissue included benign squamous epithelium (6), dysplastic squamous epithelium (4), and carcinoma in situ (5). Three (50%) of the samples of adjacent benign squamous epithelium had SULF2 staining, mostly in the basal to middle layers with weak to moderate intensity. One (25%) of the samples of dysplastic squamous mucosa demonstrated SULF-2 staining. Interestingly, the area with low-

grade dysplasia had weak intensity, and the area with high-grade dysplasia had moderate intensity. All 5 (100%) of the samples of carcinoma in situ had SULF2 staining, mostly in the basal layer with moderate intensity.

Survival analysis

Median follow-up time was 53.6 months (inter-quartile range, 15.0 to 97.6 months). Fifty-five patients died, including 38/75 (51%) patients with adenocarcinoma and 17/25 (68%) patients with squamous cell carcinoma.

In the univariate Cox proportional hazards models, pathologic stage, neoadjuvant therapy, and histologic type were significantly associated with overall survival; these were included in the multivariate model. Year of surgery and histologic grade were not significantly associated with survival; these were not included in the multivariate model. For every 10% increase in the percentage of tumor cells staining for SULF2, the risk of death increased by 4%, but this effect was not significant (p=0.42). Unadjusted Kaplan-Meier survival estimates confirmed these results (Figure 4).

In the multivariate Cox proportional hazards model, histologic type was no longer associated with overall survival (Table 2). Higher stage was still associated with worse survival (p=0.001), and patients who underwent neoadjuvant therapy still had a higher risk of death compared to those who did not (p=0.003). The percentage of tumor cells staining was significantly associated with overall survival. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increased by 13% (p=0.03).

Table 2. Univariate and multivariate Cox proportional hazards models for overall survival.

	Univariate Analysis			**Multivariate Analysis**		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
Age (per 10 years)	1.09	0.99-1.03	0.46	1.19	0.92-1.54	0.19
Sex						
Male	1			1		
Female	1.17	0.61-2.21	0.64	1.30	0.61-2.78	0.50
Race	1	0.22-3.77	0.59	1	0.18-4.49	0.40

White	0.91	0.53-9.17		0.91	0.80-17.57	
Asian	2.21		0.90	3.76		0.90
Black			0.27			0.09
Histologic type						
Adenocarcinoma	1			1		
Squamous cell	1.63	0.92-2.88	0.10	1.14	0.53-2.43	0.74
Pathologic stage			0.004			0.001
I	1			1		
II	2.42	1.08-5.38	0.03	2.65	1.13-6.22	0.03
III	4.01	1.88-8.55	<0.0005	5.14	2.23-11.82	<0.0005
IV	2.14	0.27-16.94	0.47	0.94	0.11-8.10	0.96
Neoadjuvant therapy						
No	1			1		
Yes	1.78	1.02-3.11	0.04	2.65	1.38-5.09	0.003
Percent (per 10%)	1.04	0.95-1.14	0.42	1.13	1.01-1.26	0.03

Only percentage, not intensity, of tumor cells staining was significantly associated with overall survival. Subgroup analysis by histologic type and neoadjuvant therapy did not show significant associations between percentage or intensity of tumor cells staining and survival.

DISCUSSION

This study showed that higher SULF2 expression by immunohistochemistry is associated with worse overall survival in esophageal cancer. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increases by 13%. As expected, higher stage was also significantly associated with worse overall survival. Interestingly, patients who underwent neoadjuvant therapy had worse overall survival than those who did not. This result likely reflects selection bias, in which patients diagnosed with more aggressive tumors are more likely to receive neoadjuvant therapy.

This study also showed that SULF2 staining differed by histologic type, likely reflecting their distinct etiologies. Squamous cell carcinoma samples had significantly higher percentage and intensity of tumor cells staining than adenocarcinoma samples. These results correspond to our previous findings in non-small cell lung cancer, in which in ten of ten squamous cell carcinoma samples showed staining for SULF-2, while zero out of ten adenocarcinoma samples showed staining in the tumor cells.[16] However, in the aforementioned study, all squamous cell carcinoma and adenocarcinoma samples showed SULF2 staining of tumor stroma cells.

The association of SULF2 and overall survival corresponds to previous findings in two other types of cancer. Lai et al. showed that increased *SULF2* transcript expression was associated with worse overall survival in patients with hepatocellular carcinoma.[15] Tessema et al. showed that *SULF2* promoter methylation was associated with improved overall survival in patients with non-small cell lung cancer.[17] Our results show that increased SULF2 at the protein level is associated with worse overall survival in a third type of cancer, consistent with an important role for this extracellular enzyme in carcinogenesis.

The *SULFs* were first discovered in a study on quail embryo development, with the human orthologs, *SULF1* and *SULF2*, cloned soon thereafter.[10] SULFs have been shown to regulate several signaling pathways by changing the sulfation status of extracellular heparan sulfate proteoglycans. Ai et al. established that the SULFs promote canonical Wnt signaling, by removing 6OS from the heparan sulfate chains of heparan sulfate proteoglycans, allowing the Wnt ligands to interact with its Frizzled receptor, leading to activation of Wnt target genes.[7]

Originally, SULFs were thought to be tumor suppressors, because forced expression of a SULF in several tumor cell lines caused reduced growth-factor signaling by HB-EGF, FGF-2, or HGF, as well as diminished tumorigenicity.[19-22] Soon, however, they were thought to play an oncogenic role, as one or both SULF genes were found to be overexpressed in subsets of multiple tumors (breast, pancreatic, hepatocellular carcinoma, head and neck, lung, multiple myeloma, and glioblastoma).[10,11,13-16,20,23-25] SULF2 in particular has been identified as a candidate cancer-causing gene in two unbiased screening studies in human breast cancer and mouse brain cancer.[26,27] Moreover, in pancreatic and lung cancer cell lines, SULF2 knockdown led to reduced proliferation and reduced growth of xenografts in mice, likely due to its effect on canonical Wnt signaling pathway.[13,16] In hepatocellular cancer cell lines, overexpression of SULF2 led to increased proliferation and migration and markedly enhanced the tumorigenicity of the cells in nude mice.[15] Similarly, a recent study showed SULF2 transcripts and protein upregulation in human malignant astrocytoma, and, using knockdown and transgenic approaches, demonstrated a SULF2-dependent increase in PDGFR signaling,

tumor cell proliferation in vitro, and tumor growth in vivo.[14] The present study is the first to investigate SULF2 in esophageal cancer.

Our results show that SULF2 was expressed in 93 of 100 human esophageal tumors. Importantly, high expression of SULF2 was associated with worse prognosis in esophageal cancer. As a secreted molecule ~~that may accumulate in blood or other body fluids~~, SULF-2 is a potential diagnostic or prognostic biomarker. Current screening programs for patients with Barrett's esophagus or severe gastroesophageal reflux are ineffective in preventing esophageal adenocarcinoma, due to the slow rate of progression and resultant low incidence of esophageal cancer.[4] The development of SULF2 as a biomarker may help identify a high-risk group that would make screening more feasible. In our study, 15 out of 20 (75%) adjacent Barrett's esophagus samples had SULF2 staining. Also, 5 out of 5 (100%) adjacent carcinoma in situ samples had SULF2 staining, although 3 out of 6 (50%) adjacent benign squamous epithelium had SULF2 staining as well. Future studies are needed to evaluate SULF2 specifically in these precursor lesions as well as in blood or other body fluids.~~serum.~~

Given the prevalence and poor prognosis of esophageal cancer, advances in chemotherapy and targeted therapies based on key molecular pathways are greatly needed**.** Although further investigations are required for validation of the role of SULF2 in esophageal cancer, our results raise the possibility that SULF2 could be a therapeutic target. SULF2 is an extracellular enzyme and thus is potentially amenable to inhibition by either antibody-based or small-molecule drugs. *SULF1* and *SULF2* double knockout mice had increased perinatal mortality, and the mice that did survive had lower body weight.[28]; however *SULF1* and *SULF2* single knockout mice appear normal and have normal survival, suggesting that each enzyme might be singly targeted.

In conclusion, we show SULF2 expression in esophageal carcinoma. The vast majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had some degree of SULF2 protein expression. Higher percentage of tumor cells staining for SULF2 is significantly associated with worse overall survival in these patients. Patients with esophageal cancer have an extremely poor prognosis, and SULF2 is a promising biomarker that could play an important role in the diagnosis and prognosis.

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Competing Interests

None

Funding

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Contributorship Statement

NSL: study design, data collection, patient selection, statistical analysis and interpretation, paper writing; MVZ: patient selection, reviewing H&E slides, scoring SULF2 stained slides; SDR, DMJ: data analysis and interpretation; HLA: study design, data analysis and interpretation, paper writing.

Data Sharing Statement

No additional data available.

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Figure Legends

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.

Figure 2. Representative sections from adenocarcinoma samples with no staining **(A)**, weak staining **(B)**, and moderate staining **(C)**; and a squamous cell carcinoma sample with strong staining **(D)**. Endothelial cell staining served as an internal positive control, as seen in the upper left corner of **(A)**.

Figure 3. SULF2 staining of adjacent Barrett's esophagus.

Figure 4. Kaplan-Meier survival estimates by **(A)** stage, **(B)** histologic type, **(C)** neoadjuvant therapy, and **(D)** percentage of tumors cells staining. P-values were calculated using the log-rank test.

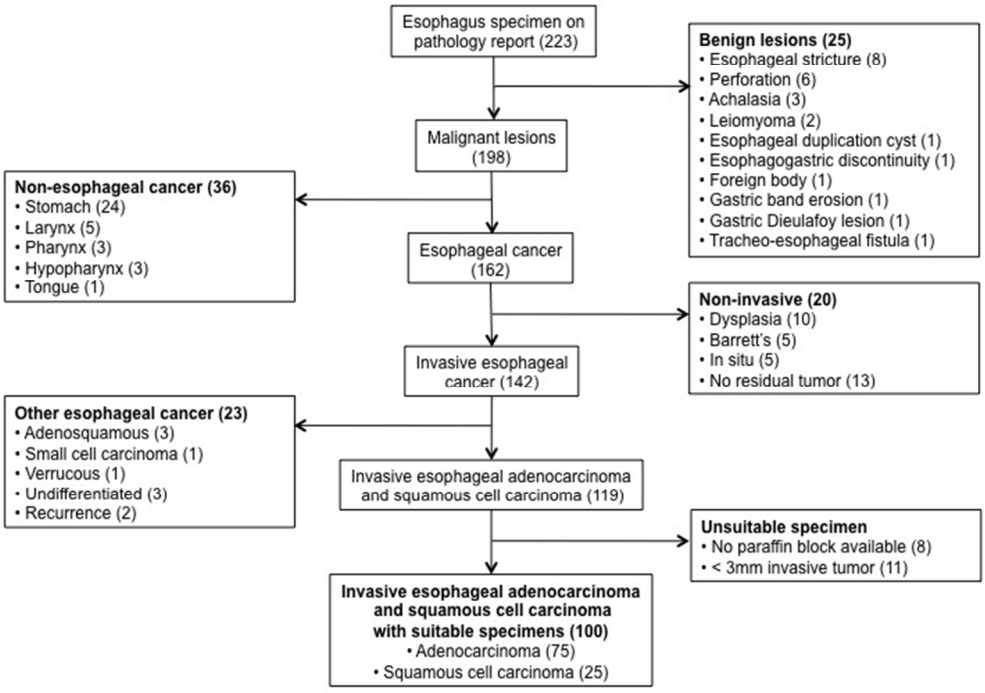

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.
254x190mm (72 x 72 DPI)

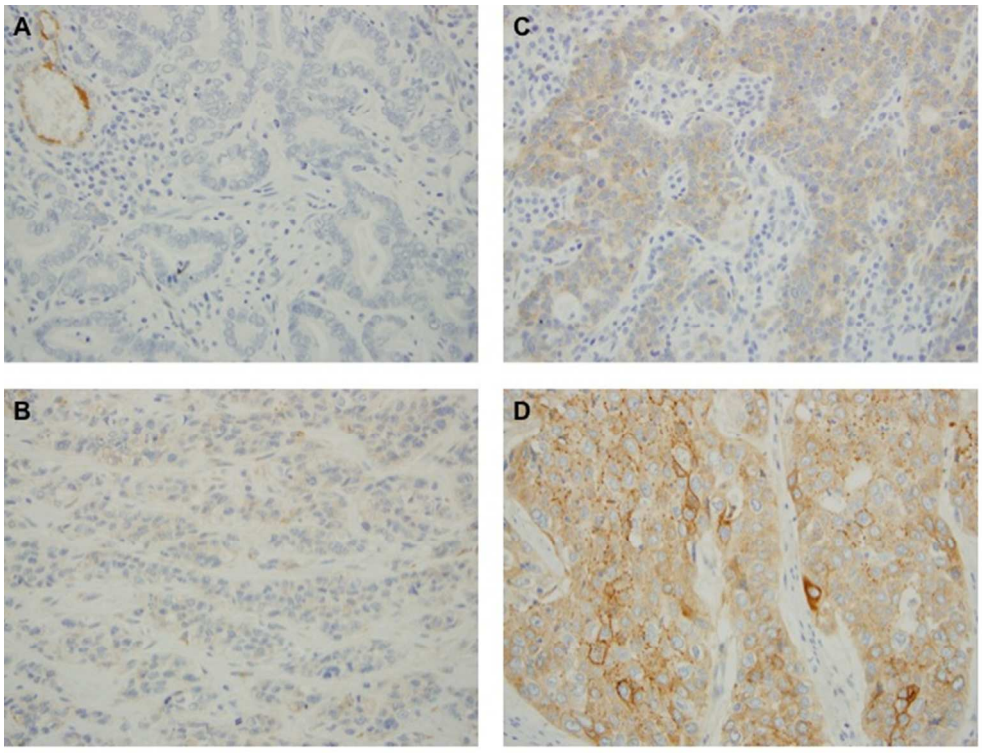

Figure 2. Representative sections from adenocarcinoma samples with no staining (A), weak staining (B), and moderate staining (C); and a squamous cell carcinoma sample with strong staining (D). Endothelial cell staining served as an internal positive control, as seen in the upper left corner of (A).
254x190mm (72 x 72 DPI)

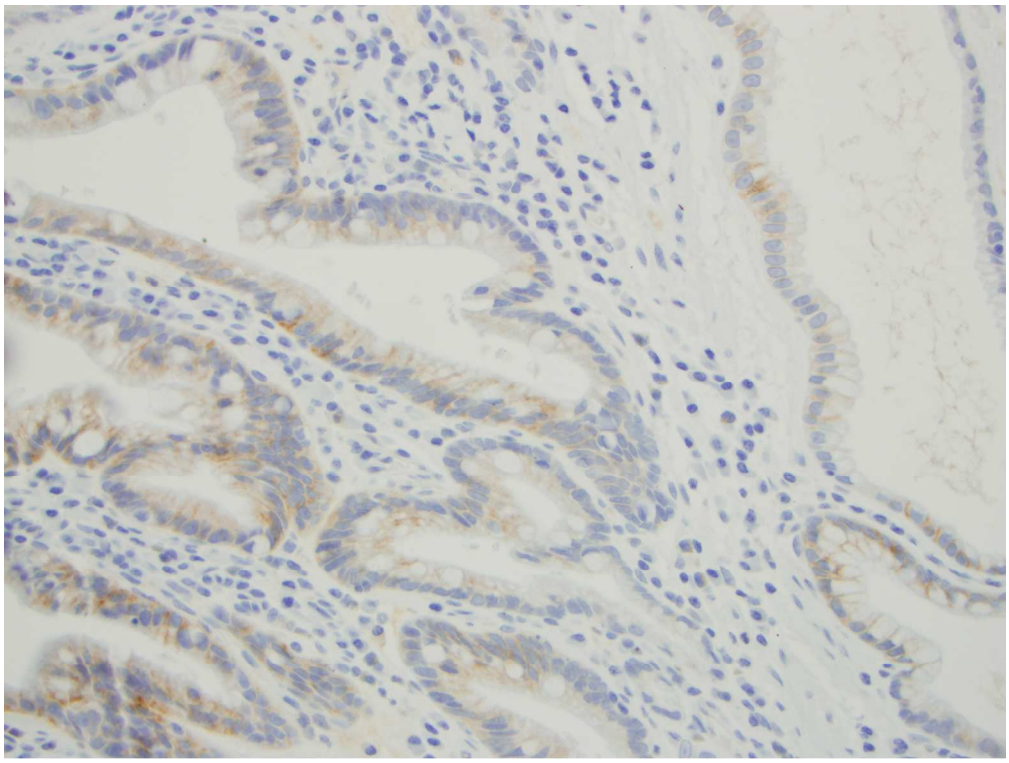

Figure 3. SULF2 staining of adjacent Barrett's esophagus.
239x180mm (216 x 216 DPI)

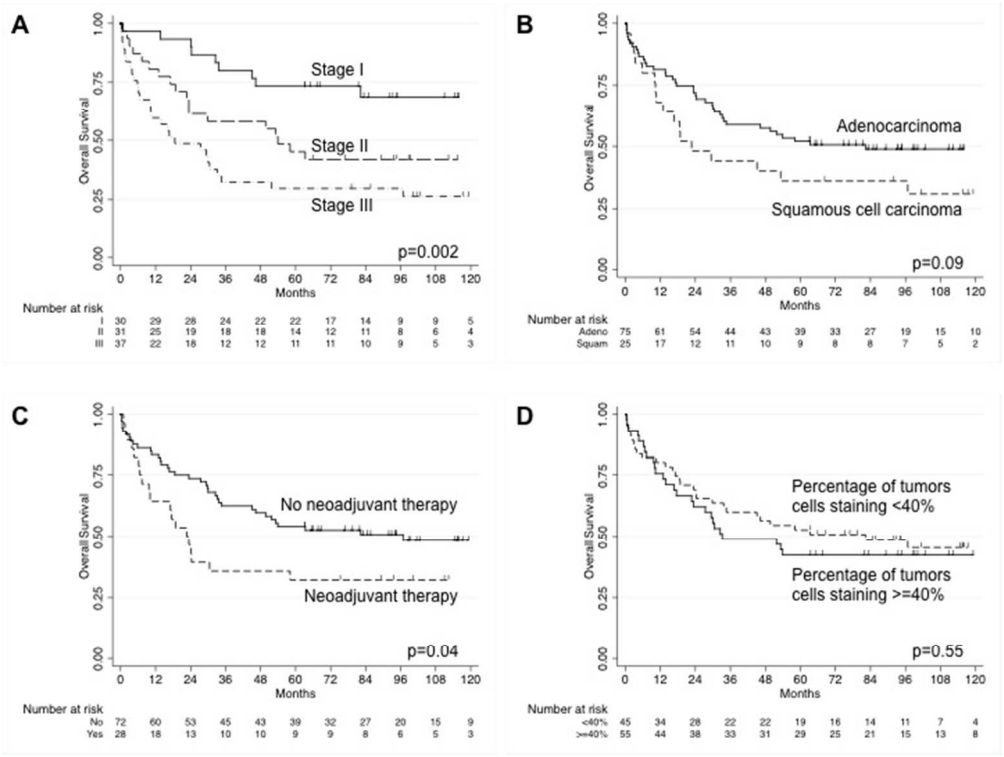

Figure 4. Kaplan-Meier survival estimates by (A) stage, (B) histologic type, (C) neoadjuvant therapy, and (D) percentage of tumors cells staining. P-values were calculated using the log-rank test.
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SULF2 expression by immunohistochemistry and overall survival in esophageal cancer

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SULF2 expression by immunohistochemistry and overall survival in esophageal cancer: a cohort study

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ABSTRACT

Objectives Esophageal cancer is the eighth most commonly diagnosed cancer worldwide, and there is a need for biomarkers to improve diagnosis, prognosis, and treatment. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in patients with esophageal cancer.

Design Cohort study

Setting Single tertiary care center

Participants We included patients who underwent esophagectomy for invasive esophageal adenocarcinoma and squamous cell carcinoma at a tertiary care center from 1997 to 2006. We excluded patients with recurrent esophageal cancer or less than 3 mm invasive tumor on H&E stained slide. A section from each paraffin-embedded tissue specimen was stained with an anti-SULF2 monoclonal antibody.

Outcome measures A pathologist blinded to overall survival determined the percentage and intensity of tumor cells staining. Vital status was obtained through the Social Security Death Master File, and overall survival was calculated from the date of surgery.

Results One-hundred patients with invasive esophageal cancer were identified, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma. The squamous cell carcinoma samples had a higher mean percentage and intensity of tumor cells staining compared to the adenocarcinoma samples. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the hazard ratio for death increased by 13% (95% confidence interval, 1.01-1.25; p=0.03).

Conclusions The majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had SULF2 staining. The percentage of tumor cells staining for SULF2 was significantly associated with overall survival. Thus, SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

ARTICLE SUMMARY

Article focus

1. Esophageal cancer is the eighth most commonly diagnosed cancer and sixth most common cause of cancer death worldwide. There is a desperate need for biomarkers to improve diagnosis, prognosis, and treatment of this disease.
2. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis in several types of cancer.
3. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in 100 patients with esophageal cancer.

Key messages

1. We show for the first time SULF2 staining in esophageal cancer, including the majority of adenocarcinoma samples and all of the squamous cell carcinoma samples.
2. The percentage of tumor cells staining for SULF2 is significantly associated with overall survival in multivariate analysis.
3. SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

Strengths and Imitations of this study

1. A major strength of this study is the use tumor samples from a large, carefully selected cohort of patients with esophageal cancer.
2. Another major strength of the study is the novelty of SULF2, which may be a hub in the network of signaling pathways critical for cancer development and progression.
3. A limitation of this study is the lack of functional data that confirm causality of SULF2 in esophageal cancer cell lines. However, the significant association between increased SULF2 expression and worse overall survival in patients with esophageal cancer justifies investigation into the role of SULF2 in esophageal cancer cells, beyond the scope of the present study.
4. Another limitation of this study is the variability of SULF2 staining across samples. While SULF2 expression by immunohistochemistry is detected in the majority of the esophageal tumors, it is possible that SULF2 will be most useful as a biomarker in a subset of patients with esophageal cancer.

INTRODUCTION

Esophageal cancer is the eighth most commonly diagnosed cancer and the sixth most common cause of cancer death worldwide.[1] There are two main histologic types, each with distinct risk factors, geographic patterns, and temporal trends. Esophageal adenocarcinoma is associated with gastroesophageal reflux disease, obesity, and the precursor lesion Barrett's esophagus; its incidence has increased faster than that of any other cancer in the United States in the past few decades.[2] Esophageal squamous cell carcinoma is associated with tobacco smoking, alcohol consumption, and poor nutrition; its incidence remains much higher than that of esophageal adenocarcinoma in most of the world.[3] Patients with esophageal cancer continue to have a poor prognosis, with five-year overall survival still less than 15%.[4] A greater understanding of the molecular basis of esophageal cancer, including the development of new biomarkers, is greatly needed to improve the diagnosis, prognosis, and treatment of patients with this disease.

Heparan sulfate proteoglycans (HSPGs) consist of core proteins that are modified by the covalent addition of heparan or chondroitin sulfate chains.[5] These chains are composed of repeating disaccharide units, which are variably sulfated at four different positions. HSPGs perform enumerable signaling functions, using their sulfated chains to bind diverse protein ligands, such as growth factors, morphogens, and cytokines. These interactions depend on the pattern of the sulfation modifications with the 6-O-sulfation of glucosamine (6OS) known to be key for binding many ligands.[6] Two recently discovered sulfatases (SULF1 and SULF2) provide a novel mechanism for the regulation of HSPG-dependent signaling by acting on 6OS on the outside of cells. Work by us and others has shown that SULFs are neutral pH, extracellular enzymes which remove 6OS from intact HSPGs; they promote key signaling pathways by mobilizing protein ligands (e.g., Wnt ligands, GDNF, and BMP-4) from HSPG sequestration, thus liberating the ligands for binding to signal transduction receptors.[7-10]

One or both SULFs are broadly overexpressed at the transcript level in many human cancers, including non-small cell lung cancer, hepatocellular carcinoma, breast cancer, head and neck cancer, pancreatic adenocarcinoma, multiple myeloma, gastric carcinoma, and glioblastomas.[11,12] SULF2 has been directly implicated as a driver of carcinogenesis in pancreatic cancer[13], murine and human glioblastoma[14], hepatocellular carcinoma[15], and non-small cell lung cancer[16]. Moreover, *SULF2* promoter methylation and expression has been associated with overall survival in lung cancer and hepatocellular carcinoma, respectively.[15,17] However, there are no reports on SULF2 expression in esophageal cancer. This study evaluated SULF2 expression by immunohistochemistry and its association with overall survival in a cohort of patients with esophageal cancer.

METHODS

Patients

We identified patients who underwent esophagectomy at the University of California, San Francisco (UCSF) during the 10-year period from 1997 to 2006. We included patients undergoing primary resection for invasive esophageal adenocarcinoma and squamous cell carcinoma. Patients undergoing salvage surgery for recurrent esophageal cancer were excluded. We evaluated cases with at least 3 mm of invasive carcinoma on histologic sections and for which corresponding paraffin blocks were available. Clinical data was obtained through review of electronic medical records. Histologic data was obtained through review of pathology reports and confirmed by review of H&E stained sections by a pathologist. Pathologic stage was determined by the 7th edition of the American Joint Committee on Cancer (AJCC) staging system.[18] Vital status was obtained through the Social Security Death Master File. Overall survival was calculated from the date of surgery. The UCSF institutional review board approved this study.

Immunohistochemistry

A 5 μm section from each paraffin-embedded tissue specimen was stained with a mouse monoclonal antibody to SULF2 (AbD Serotec MCA5692T or Novus Biologicals NBP1-36727)[16] at a concentration of 2 μg/ml with avidin-biotin blocking. A pathologist blinded to patient outcome determined the percentage and intensity of tumor cells staining. The percentage of tumor cells staining was scored from 0 to 100%. The pathologist evaluated all of the tumor cells on each slide, so the number of cells evaluated per slide depended on the size of the tumor and varied widely. The intensity of tumor cells staining was assessed at 100x magnification and scored from 0 to 3. A score of 0 represented no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. When specimens showed a range of intensity, the mean intensity was recorded. The presence of endothelial cell staining was assessed for each slide and functioned as an internal positive control. Staining of tumor-associated stroma was also noted.

Statistical Analysis

Patient baseline characteristics and immunohistochemistry scores were summarized and compared by histologic type, using the Student's t-test for continuous variables and the chi-squared test for categorical variables. Survival analysis was performed using univariate and multivariate Cox proportional hazards models. Age, sex, and race were included in the multivariate model a priori. Histologic type, stage, grade, neoadjuvant therapy, and year of operation were included in the multivariate analysis only if the p-value was less than 0.10 in the univariate analysis. We repeated our analyses in pre-specified subgroups by histologic type (adenocarcinoma and squamous cell carcinoma) and neoadjuvant therapy (yes and no). In order to account for the possible misdiagnosis of gastric adenocarcinoma arising at the

gastroesophageal junction as esophageal adenocarcinoma, we also repeated our analyses in the subgroup of patients with adenocarcinoma, excluding those with tumors located at the gastroesophageal junction and not associated with Barrett's esophagus. For all statistical tests, a two-sided alpha level of 0.05 was considered statistically significant. Analyses were performed using Stata version 11.

RESULTS

Patients

We identified 233 patients who underwent esophagectomy at UCSF during the 10-year period from 1997 to 2006 (Figure 1). Of these, 100 patients met our inclusion criteria, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma.

There were several differences in baseline characteristics between patients with adenocarcinoma and those with squamous cell carcinoma (Table 1). Patients with adenocarcinoma had a higher proportion of men (82% vs. 52%; p<0.0005). Adenocarcinomas were more often located at the gastroesophageal junction and associated with Barrett's esophagus. Patients with adenocarcinoma had a higher frequency of neoadjuvant therapy and were generally diagnosed and resected at an earlier pathologic stage. There was no difference in age, race, or ethnicity between histologic types.

Table 1. Patient baseline characteristics.

	All patients (N=100)	**Patients with AC (N=75)**	**Patients with SCC (N=25)**	**p-value†**
Age, mean ± SD—years	64.2 ± 11.6	63.9 ± 11.0	65.2 ± 13.5	0.63
Sex—no. (%)				<0.0005
Female	21 (21)	9 (12)	12 (48)	
Male	79 (79)	66 (88)	13 (52)	
Race—no. (%)	80 (80)	62 (84)	18 (72)	0.13
White	4 (4)	1 (1)	3 (12)	
Asian	3 (3)	2 (3)	1 (4)	

Black	13 (13)	10 (13)	3 (12)	
Missing				
Ethnicity—no. (%)				0.48
Non-Hispanic	90 (90)	66 (88)	24 (96)	
Hispanic	2 (2)	2 (3)	0	
Missing	8 (8)	7 (9)	1 (4)	
Location of tumor‡—no. (%)				<0.0005
Upper esophagus	5 (5)	0	5 (20)	
Middle esophagus	9 (9)	2 (3)	7 (28)	
Lower esophagus	31 (31)	20 (27)	11 (44)	
Gastro-esophageal junction	55 (55)	53 (71)	2 (8)	
Presence of Barrett's esophagus—no. (%)				<0.0005
Yes	46 (46)	46 (61)	0	
No	54 (54)	29 (39)	25 (100)	
Pathologic stage—no. (%)				0.06
I	30 (30)	27 (36)	3 (12)	
II	31 (31)	23 (31)	8 (32)	
III	37 (37)	23 (31)	14 (56)	
IV	2 (2)	2 (3)	0	
Histologic grade—no. (%)				0.10
1 (Well-differentiated)	11	11	0	
2 (Moderately differentiated)	41	30	11	
3 (Poorly differentiated)	44	30	14	
4 (Undifferentiated)	4	4	0	

Neoadjuvant therapy				0.04
Yes	28 (28)	25 (33)	3 (12)	
No	72 (72)	50 (67)	22 (88)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; SD, standard deviation.

† P-values were calculated using the t-test for continuous variables and the chi-squared test for categorical variables.

‡ Location of tumor was recorded as the most superior location of the tumor in the esophagus. For example, if the pathology report noted tumor at the upper and middle esophagus, the location was recorded as upper esophagus.

Immunohistochemistry

SULF2 staining was detected on 93/100 of the specimens, including 68/75 (91%) adenocarcinoma samples and 25/25 (100%) squamous cell carcinoma samples (Figure 2). All samples had endothelial cell staining, which served as an internal positive control.

The squamous cell carcinoma samples had a higher mean percentage (49% vs. 36%; p=0.06) and intensity (1.9 vs. 1.4; p=0.0007) of tumor cells staining compared to the adenocarcinoma samples. Samples from patients who underwent neoadjuvant therapy had a lower mean intensity of tumor cells staining (1.3 vs. 1.6; p=0.03), but this difference was not evident when patients were stratified by histologic type. Neither percentage nor intensity of tumor cells staining was significantly associated with the other clinical and pathologic variables, including stage and grade.

SULF2 staining was present in some adjacent tissues. Among the 75 adenocarcinoma samples, incidental adjacent tissue included squamous epithelium (33), gastric epithelium (24), and Barrett's esophagus (20). Seventeen (52%) of the samples of adjacent benign squamous epithelium showed SULF2 staining of this epithelium. Staining was generally focal in the basal layer with weak to moderate intensity. Seven (29%) of the samples of adjacent benign gastric epithelium showed SULF2 staining of this epithelium, mostly patchy in the basal layer with weak to moderate intensity. Fifteen (75%) of the samples of Barrett's esophagus showed SULF2 staining of this metaplastic epithelium, mostly patchy with weak to moderate intensity (Figure 3).

Among the 25 squamous cell carcinoma cases, incidental adjacent tissue included benign squamous epithelium (6), dysplastic squamous epithelium (4), and carcinoma in situ (5). Three (50%) of the samples of adjacent benign squamous epithelium had SULF2 staining, mostly in the basal to middle layers with weak to moderate intensity. One (25%) of the samples of dysplastic squamous mucosa demonstrated SULF2 staining. Interestingly, the area with low-

grade dysplasia had weak intensity, and the area with high-grade dysplasia had moderate intensity. All 5 (100%) of the samples of carcinoma in situ had SULF2 staining, mostly in the basal layer with moderate intensity.

Survival analysis

Median follow-up time was 53.6 months (inter-quartile range, 15.0 to 97.6 months). Fifty-five patients died, including 38/75 (51%) patients with adenocarcinoma and 17/25 (68%) patients with squamous cell carcinoma.

In the univariate Cox proportional hazards models, pathologic stage, neoadjuvant therapy, and histologic type were significantly associated with overall survival; these were included in the multivariate model. Year of surgery and histologic grade were not significantly associated with survival; these were not included in the multivariate model. For every 10% increase in the percentage of tumor cells staining for SULF2, the risk of death increased by 4%, but this effect was not significant (p=0.42). Unadjusted Kaplan-Meier survival estimates confirmed these results (Figure 4).

In the multivariate Cox proportional hazards model, histologic type was no longer associated with overall survival (Table 2). Higher stage was still associated with worse survival (p=0.001), and patients who underwent neoadjuvant therapy still had a higher risk of death compared to those who did not (p=0.003). The percentage of tumor cells staining was significantly associated with overall survival. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increased by 13% (p=0.03).

Table 2. Univariate and multivariate Cox proportional hazards models for overall survival.

	Univariate Analysis			**Multivariate Analysis**		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
Age (per 10 years)	1.09	0.99-1.03	0.46	1.19	0.92-1.54	0.19
Sex						
Male	1			1		
Female	1.17	0.61-2.21	0.64	1.30	0.61-2.78	0.50
Race	1	0.22-3.77	0.59	1	0.18-4.49	0.40

White	0.91	0.53-9.17		0.91	0.80-17.57	
Asian	2.21		0.90	3.76		0.90
Black			0.27			0.09
Histologic type						
Adenocarcinoma	1			1		
Squamous cell	1.63	0.92-2.88	0.10	1.14	0.53-2.43	0.74
Pathologic stage			0.004			0.001
I	1			1		
II	2.42	1.08-5.38	0.03	2.65	1.13-6.22	0.03
III	4.01	1.88-8.55	<0.0005	5.14	2.23-11.82	<0.0005
IV	2.14	0.27-16.94	0.47	0.94	0.11-8.10	0.96
Neoadjuvant therapy						
No	1			1		
Yes	1.78	1.02-3.11	0.04	2.65	1.38-5.09	0.003
Percent (per 10%)	1.04	0.95-1.14	0.42	1.13	1.01-1.26	0.03

Only percentage, not intensity, of tumor cells staining was significantly associated with overall survival. Subgroup analysis by histologic type and neoadjuvant therapy did not show significant associations between percentage or intensity of tumor cells staining and survival.

DISCUSSION

This study showed that higher SULF2 expression by immunohistochemistry is associated with worse overall survival in esophageal cancer. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increases by 13%. As expected, higher stage was also significantly associated with worse overall survival. Interestingly, patients who underwent neoadjuvant therapy had worse overall survival than those who did not. This result likely reflects selection bias, in which patients diagnosed with more aggressive tumors are more likely to receive neoadjuvant therapy.

This study also showed that SULF2 staining differed by histologic type, likely reflecting their distinct etiologies. Squamous cell carcinoma samples had significantly higher percentage and intensity of tumor cells staining than adenocarcinoma samples. These results correspond to our previous findings in non-small cell lung cancer, in which in ten of ten squamous cell carcinoma samples showed staining for SULF2, while zero out of ten adenocarcinoma samples showed staining in the tumor cells.[16] However, in the aforementioned study, all squamous cell carcinoma and adenocarcinoma samples showed SULF2 staining of tumor stroma cells.

The association of SULF2 and overall survival corresponds to previous findings in two other types of cancer. Lai et al. showed that increased *SULF2* transcript expression was associated with worse overall survival in patients with hepatocellular carcinoma.[15] Tessema et al. showed that *SULF2* promoter methylation was associated with improved overall survival in patients with non-small cell lung cancer.[17] Our results show that increased SULF2 at the protein level is associated with worse overall survival in a third type of cancer, consistent with an important role for this extracellular enzyme in carcinogenesis.

The *SULFs* were first discovered in a study on quail embryo development, with the human orthologs, *SULF1* and *SULF2*, cloned soon thereafter.[10] SULFs have been shown to regulate several signaling pathways by changing the sulfation status of extracellular heparan sulfate proteoglycans. Ai et al. established that the SULFs promote canonical Wnt signaling, by removing 6OS from the heparan sulfate chains of heparan sulfate proteoglycans, allowing the Wnt ligands to interact with its Frizzled receptor, leading to activation of Wnt target genes.[7]

Originally, SULFs were thought to be tumor suppressors, because forced expression of a SULF in several tumor cell lines caused reduced growth-factor signaling by HB-EGF, FGF-2, or HGF, as well as diminished tumorigenicity.[19-22] Soon, however, they were thought to play an oncogenic role, as one or both SULF genes were found to be overexpressed in subsets of multiple tumors (breast, pancreatic, hepatocellular carcinoma, head and neck, lung, multiple myeloma, and glioblastoma).[10,11,13-16,20,23-25] SULF2 in particular has been identified as a candidate cancer-causing gene in two unbiased screening studies in human breast cancer and mouse brain cancer.[26,27] Moreover, in pancreatic and lung cancer cell lines, SULF2 knockdown led to reduced proliferation and reduced growth of xenografts in mice, likely due to its effect on canonical Wnt signaling pathway.[13,16] In hepatocellular cancer cell lines, overexpression of SULF2 led to increased proliferation and migration and markedly enhanced the tumorigenicity of the cells in nude mice.[15] Similarly, a recent study showed SULF2 transcripts and protein upregulation in human malignant astrocytoma, and, using knockdown and transgenic approaches, demonstrated a SULF2-dependent increase in PDGFR signaling,

tumor cell proliferation in vitro, and tumor growth in vivo.[14] The present study is the first to investigate SULF2 in esophageal cancer.

Our results show that SULF2 was expressed in 93 of 100 human esophageal tumors. Importantly, high expression of SULF2 was associated with worse prognosis in esophageal cancer. As a secreted molecule, SULF2 is a potential diagnostic or prognostic biomarker. Current screening programs for patients with Barrett's esophagus or severe gastroesophageal reflux are ineffective in preventing esophageal adenocarcinoma, due to the slow rate of progression and resultant low incidence of esophageal cancer.[4] The development of SULF2 as a biomarker may help identify a high-risk group that would make screening more feasible. In our study, 15 out of 20 (75%) adjacent Barrett's esophagus samples had SULF2 staining. Also, 5 out of 5 (100%) adjacent carcinoma in situ samples had SULF2 staining, although 3 out of 6 (50%) adjacent benign squamous epithelium had SULF2 staining as well. Future studies are needed to evaluate SULF2 specifically in these precursor lesions as well as in blood or other body fluids.

Given the prevalence and poor prognosis of esophageal cancer, advances in chemotherapy and targeted therapies based on key molecular pathways are greatly needed**.** Although further investigations are required for validation of the role of SULF2 in esophageal cancer, our results raise the possibility that SULF2 could be a therapeutic target. SULF2 is an extracellular enzyme and thus is potentially amenable to inhibition by either antibody-based or small-molecule drugs. *SULF1* and *SULF2* double knockout mice had increased perinatal mortality, and the mice that did survive had lower body weight.[28]; however *SULF1* and *SULF2* single knockout mice appear normal and have normal survival, suggesting that each enzyme might be singly targeted.

In conclusion, we show SULF2 expression in esophageal carcinoma. The vast majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had some degree of SULF2 protein expression. Higher percentage of tumor cells staining for SULF2 is significantly associated with worse overall survival in these patients. Patients with esophageal cancer have an extremely poor prognosis, and SULF2 is a promising biomarker that could play an important role in the diagnosis and prognosis.

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Competing Interests

None

Funding

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Contributorship Statement

NSL: study design, data collection, patient selection, statistical analysis and interpretation, paper writing; MVZ: patient selection, reviewing H&E slides, scoring SULF2 stained slides; SDR, DMJ: data analysis and interpretation; HLA: study design, data analysis and interpretation, paper writing.

Data Sharing Statement

No additional data available.

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Figure Legends

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.

Figure 2. Representative sections from adenocarcinoma samples with no staining **(A)**, weak staining **(B)**, and moderate staining **(C)**; and a squamous cell carcinoma sample with strong staining **(D)**. Endothelial cell staining served as an internal positive control, as seen in the upper left corner of **(A)**.

Figure 3. SULF2 staining of adjacent Barrett's esophagus.

Figure 4. Kaplan-Meier survival estimates by **(A)** stage, **(B)** histologic type, **(C)** neoadjuvant therapy, and **(D)** percentage of tumors cells staining. P-values were calculated using the log-rank test.

SULF2 expression by immunohistochemistry and overall survival in esophageal cancer: a cohort study

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ABSTRACT

Objectives Esophageal cancer is the eighth most commonly diagnosed cancer worldwide, and there is a need for biomarkers to improve diagnosis, prognosis, and treatment. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in patients with esophageal cancer.

Design Cohort study

Setting Single tertiary care center

Participants We included patients who underwent esophagectomy for invasive esophageal adenocarcinoma and squamous cell carcinoma at a tertiary care center from 1997 to 2006. We excluded patients with recurrent esophageal cancer or less than 3 mm invasive tumor on H&E stained slide. A section from each paraffin-embedded tissue specimen was stained with an anti-SULF2 monoclonal antibody.

Outcome measures A pathologist blinded to overall survival determined the percentage and intensity of tumor cells staining. Vital status was obtained through the Social Security Death Master File, and overall survival was calculated from the date of surgery.

Results One-hundred patients with invasive esophageal cancer were identified, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma. The squamous cell carcinoma samples had a higher mean percentage and intensity of tumor cells staining compared to the adenocarcinoma samples. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the hazard ratio for death increased by 13% (95% confidence interval, 1.01-1.25; p=0.03).

Conclusions The majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had SULF2 staining. The percentage of tumor cells staining for SULF2 was significantly associated with overall survival. Thus, SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

ARTICLE SUMMARY

Article focus

1. Esophageal cancer is the eighth most commonly diagnosed cancer and sixth most common cause of cancer death worldwide. There is a desperate need for biomarkers to improve diagnosis, prognosis, and treatment of this disease.
2. SULF2 is an extracellular endosulfatase that regulates several signaling pathways in carcinogenesis and has been associated with poor prognosis in several types of cancer.
3. This study evaluates the relationship between SULF2 expression by immunohistochemistry and overall survival in 100 patients with esophageal cancer.

Key messages

1. We show for the first time SULF2 staining in esophageal cancer, including the majority of adenocarcinoma samples and all of the squamous cell carcinoma samples.
2. The percentage of tumor cells staining for SULF2 is significantly associated with overall survival in multivariate analysis.
3. SULF2 is a potential biomarker in esophageal cancer and may have an important role in the management of patients with this disease.

Strengths and Imitations of this study

1. A major strength of this study is the use tumor samples from a large, carefully selected cohort of patients with esophageal cancer.
2. Another major strength of the study is the novelty of SULF2, which may be a hub in the network of signaling pathways critical for cancer development and progression.
3. A limitation of this study is the lack of functional data that confirm causality of SULF2 in esophageal cancer cell lines. However, the significant association between increased SULF2 expression and worse overall survival in patients with esophageal cancer justifies investigation into the role of SULF2 in esophageal cancer cells, beyond the scope of the present study.
4. Another limitation of this study is the variability of SULF2 staining across samples. While SULF2 expression by immunohistochemistry is detected in the majority of the esophageal tumors, it is possible that SULF2 will be most useful as a biomarker in a subset of patients with esophageal cancer.

INTRODUCTION

Esophageal cancer is the eighth most commonly diagnosed cancer and the sixth most common cause of cancer death worldwide.[1] There are two main histologic types, each with distinct risk factors, geographic patterns, and temporal trends. Esophageal adenocarcinoma is associated with gastroesophageal reflux disease, obesity, and the precursor lesion Barrett's esophagus; its incidence has increased faster than that of any other cancer in the United States in the past few decades.[2] Esophageal squamous cell carcinoma is associated with tobacco smoking, alcohol consumption, and poor nutrition; its incidence remains much higher than that of esophageal adenocarcinoma in most of the world.[3] Patients with esophageal cancer continue to have a poor prognosis, with five-year overall survival still less than 15%.[4] A greater understanding of the molecular basis of esophageal cancer, including the development of new biomarkers, is greatly needed to improve the diagnosis, prognosis, and treatment of patients with this disease.

Heparan sulfate proteoglycans (HSPGs) consist of core proteins that are modified by the covalent addition of heparan or chondroitin sulfate chains.[5] These chains are composed of repeating disaccharide units, which are variably sulfated at four different positions. HSPGs perform enumerable signaling functions, using their sulfated chains to bind diverse protein ligands, such as growth factors, morphogens, and cytokines. These interactions depend on the pattern of the sulfation modifications with the 6-O-sulfation of glucosamine (6OS) known to be key for binding many ligands.[6] Two recently discovered sulfatases (SULF1 and SULF2) provide a novel mechanism for the regulation of HSPG-dependent signaling by acting on 6OS on the outside of cells. Work by us and others has shown that SULFs are neutral pH, extracellular enzymes which remove 6OS from intact HSPGs; they promote key signaling pathways by mobilizing protein ligands (e.g., Wnt ligands, GDNF, and BMP-4) from HSPG sequestration, thus liberating the ligands for binding to signal transduction receptors.[7-10]

One or both SULFs are broadly overexpressed at the transcript level in many human cancers, including non-small cell lung cancer, hepatocellular carcinoma, breast cancer, head and neck cancer, pancreatic adenocarcinoma, multiple myeloma, gastric carcinoma, and glioblastomas.[11,12] SULF2 has been directly implicated as a driver of carcinogenesis in pancreatic cancer[13], murine and human glioblastoma[14], hepatocellular carcinoma[15], and non-small cell lung cancer[16]. Moreover, *SULF2* promoter methylation and expression has been associated with overall survival in lung cancer and hepatocellular carcinoma, respectively.[15,17] However, there are no reports on SULF2 expression in esophageal cancer. This study evaluated SULF2 expression by immunohistochemistry and its association with overall survival in a cohort of patients with esophageal cancer.

METHODS

Patients

We identified patients who underwent esophagectomy at the University of California, San Francisco (UCSF) during the 10-year period from 1997 to 2006. We included patients undergoing primary resection for invasive esophageal adenocarcinoma and squamous cell carcinoma. Patients undergoing salvage surgery for recurrent esophageal cancer were excluded. We evaluated cases with at least 3 mm of invasive carcinoma on histologic sections and for which corresponding paraffin blocks were available. Clinical data was obtained through review of electronic medical records. Histologic data was obtained through review of pathology reports and confirmed by review of H&E stained sections by a pathologist. Pathologic stage was determined by the 7th edition of the American Joint Committee on Cancer (AJCC) staging system.[18] Vital status was obtained through the Social Security Death Master File. Overall survival was calculated from the date of surgery. The UCSF institutional review board approved this study.

Immunohistochemistry

A 5 μm section from each paraffin-embedded tissue specimen was stained with a mouse monoclonal antibody to SULF2 (AbD Serotec MCA5692T or Novus Biologicals NBP1-36727)[16] at a concentration of 2 μg/ml with avidin-biotin blocking. A pathologist blinded to patient outcome determined the percentage and intensity of tumor cells staining. The percentage of tumor cells staining was scored from 0 to 100%. The pathologist evaluated all of the tumor cells on each slide, so the number of cells evaluated per slide depended on the size of the tumor and varied widely. The intensity of tumor cells staining was assessed at 100x magnification and scored from 0 to 3. A score of 0 represented no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. When specimens showed a range of intensity, the mean intensity was recorded. The presence of endothelial cell staining was assessed for each slide and functioned as an internal positive control. Staining of tumor-associated stroma was also noted.

Statistical Analysis

Patient baseline characteristics and immunohistochemistry scores were summarized and compared by histologic type, using the Student's t-test for continuous variables and the chi-squared test for categorical variables. Survival analysis was performed using univariate and multivariate Cox proportional hazards models. Age, sex, and race were included in the multivariate model a priori. Histologic type, stage, grade, neoadjuvant therapy, and year of operation were included in the multivariate analysis only if the p-value was less than 0.10 in the univariate analysis. We repeated our analyses in pre-specified subgroups by histologic type (adenocarcinoma and squamous cell carcinoma) and neoadjuvant therapy (yes and no). In order to account for the possible misdiagnosis of gastric adenocarcinoma arising at the

gastroesophageal junction as esophageal adenocarcinoma, we also repeated our analyses in the subgroup of patients with adenocarcinoma, excluding those with tumors located at the gastroesophageal junction and not associated with Barrett's esophagus. For all statistical tests, a two-sided alpha level of 0.05 was considered statistically significant. Analyses were performed using Stata version 11.

RESULTS

Patients

We identified 233 patients who underwent esophagectomy at UCSF during the 10-year period from 1997 to 2006 (Figure 1). Of these, 100 patients met our inclusion criteria, including 75 patients with adenocarcinoma and 25 patients with squamous cell carcinoma.

There were several differences in baseline characteristics between patients with adenocarcinoma and those with squamous cell carcinoma (Table 1). Patients with adenocarcinoma had a higher proportion of men (82% vs. 52%; p<0.0005). Adenocarcinomas were more often located at the gastroesophageal junction and associated with Barrett's esophagus. Patients with adenocarcinoma had a higher frequency of neoadjuvant therapy and were generally diagnosed and resected at an earlier pathologic stage. There was no difference in age, race, or ethnicity between histologic types.

Table 1. Patient baseline characteristics.

	All patients (N=100)	**Patients with AC (N=75)**	**Patients with SCC (N=25)**	**p-value†**
Age, mean ± SD—years	64.2 ± 11.6	63.9 ± 11.0	65.2 ± 13.5	0.63
Sex—no. (%)				<0.0005
Female	21 (21)	9 (12)	12 (48)	
Male	79 (79)	66 (88)	13 (52)	
Race—no. (%)	80 (80)	62 (84)	18 (72)	0.13
White	4 (4)	1 (1)	3 (12)	
Asian	3 (3)	2 (3)	1 (4)	

Black	13 (13)	10 (13)	3 (12)	
Missing				
Ethnicity—no. (%)				0.48
Non-Hispanic	90 (90)	66 (88)	24 (96)	
Hispanic	2 (2)	2 (3)	0	
Missing	8 (8)	7 (9)	1 (4)	
Location of tumor‡—no. (%)				<0.0005
Upper esophagus	5 (5)	0	5 (20)	
Middle esophagus	9 (9)	2 (3)	7 (28)	
Lower esophagus	31 (31)	20 (27)	11 (44)	
Gastro-esophageal junction	55 (55)	53 (71)	2 (8)	
Presence of Barrett's esophagus—no. (%)				<0.0005
Yes	46 (46)	46 (61)	0	
No	54 (54)	29 (39)	25 (100)	
Pathologic stage—no. (%)				0.06
I	30 (30)	27 (36)	3 (12)	
II	31 (31)	23 (31)	8 (32)	
III	37 (37)	23 (31)	14 (56)	
IV	2 (2)	2 (3)	0	
Histologic grade—no. (%)				0.10
1 (Well-differentiated)	11	11	0	
2 (Moderately differentiated)	41	30	11	
3 (Poorly differentiated)	44	30	14	
4 (Undifferentiated)	4	4	0	

Neoadjuvant therapy				0.04
Yes	28 (28)	25 (33)	3 (12)	
No	72 (72)	50 (67)	22 (88)	

AC, adenocarcinoma; SCC, squamous cell carcinoma; SD, standard deviation.

† P-values were calculated using the t-test for continuous variables and the chi-squared test for categorical variables.

‡ Location of tumor was recorded as the most superior location of the tumor in the esophagus. For example, if the pathology report noted tumor at the upper and middle esophagus, the location was recorded as upper esophagus.

Immunohistochemistry

SULF2 staining was detected on 93/100 of the specimens, including 68/75 (91%) adenocarcinoma samples and 25/25 (100%) squamous cell carcinoma samples (Figure 2). All samples had endothelial cell staining, which served as an internal positive control.

The squamous cell carcinoma samples had a higher mean percentage (49% vs. 36%; p=0.06) and intensity (1.9 vs. 1.4; p=0.0007) of tumor cells staining compared to the adenocarcinoma samples. Samples from patients who underwent neoadjuvant therapy had a lower mean intensity of tumor cells staining (1.3 vs. 1.6; p=0.03), but this difference was not evident when patients were stratified by histologic type. Neither percentage nor intensity of tumor cells staining was significantly associated with the other clinical and pathologic variables, including stage and grade.

SULF2 staining was present in some adjacent tissues. Among the 75 adenocarcinoma samples, incidental adjacent tissue included squamous epithelium (33), gastric epithelium (24), and Barrett's esophagus (20). Seventeen (52%) of the samples of adjacent benign squamous epithelium showed SULF2 staining of this epithelium. Staining was generally focal in the basal layer with weak to moderate intensity. Seven (29%) of the samples of adjacent benign gastric epithelium showed SULF2 staining of this epithelium, mostly patchy in the basal layer with weak to moderate intensity. Fifteen (75%) of the samples of Barrett's esophagus showed SULF2 staining of this metaplastic epithelium, mostly patchy with weak to moderate intensity (Figure 3).

Among the 25 squamous cell carcinoma cases, incidental adjacent tissue included benign squamous epithelium (6), dysplastic squamous epithelium (4), and carcinoma in situ (5). Three (50%) of the samples of adjacent benign squamous epithelium had SULF2 staining, mostly in the basal to middle layers with weak to moderate intensity. One (25%) of the samples of dysplastic squamous mucosa demonstrated SULF2 staining. Interestingly, the area with low-

grade dysplasia had weak intensity, and the area with high-grade dysplasia had moderate intensity. All 5 (100%) of the samples of carcinoma in situ had SULF2 staining, mostly in the basal layer with moderate intensity.

Survival analysis

Median follow-up time was 53.6 months (inter-quartile range, 15.0 to 97.6 months). Fifty-five patients died, including 38/75 (51%) patients with adenocarcinoma and 17/25 (68%) patients with squamous cell carcinoma.

In the univariate Cox proportional hazards models, pathologic stage, neoadjuvant therapy, and histologic type were significantly associated with overall survival; these were included in the multivariate model. Year of surgery and histologic grade were not significantly associated with survival; these were not included in the multivariate model. For every 10% increase in the percentage of tumor cells staining for SULF2, the risk of death increased by 4%, but this effect was not significant (p=0.42). Unadjusted Kaplan-Meier survival estimates confirmed these results (Figure 4).

In the multivariate Cox proportional hazards model, histologic type was no longer associated with overall survival (Table 2). Higher stage was still associated with worse survival (p=0.001), and patients who underwent neoadjuvant therapy still had a higher risk of death compared to those who did not (p=0.003). The percentage of tumor cells staining was significantly associated with overall survival. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increased by 13% (p=0.03).

Table 2. Univariate and multivariate Cox proportional hazards models for overall survival.

	Univariate Analysis			**Multivariate Analysis**		
	Hazard Ratio	**95% CI**	**p-value**	**Hazard Ratio**	**95% CI**	**p-value**
Age (per 10 years)	1.09	0.99-1.03	0.46	1.19	0.92-1.54	0.19
Sex						
Male	1			1		
Female	1.17	0.61-2.21	0.64	1.30	0.61-2.78	0.50
Race	1	0.22-3.77	0.59	1	0.18-4.49	0.40

White	0.91	0.53-9.17		0.91	0.80-17.57	
Asian	2.21		0.90	3.76		0.90
Black			0.27			0.09
Histologic type						
Adenocarcinoma	1			1		
Squamous cell	1.63	0.92-2.88	0.10	1.14	0.53-2.43	0.74
Pathologic stage			0.004			0.001
I	1			1		
II	2.42	1.08-5.38	0.03	2.65	1.13-6.22	0.03
III	4.01	1.88-8.55	<0.0005	5.14	2.23-11.82	<0.0005
IV	2.14	0.27-16.94	0.47	0.94	0.11-8.10	0.96
Neoadjuvant therapy						
No	1			1		
Yes	1.78	1.02-3.11	0.04	2.65	1.38-5.09	0.003
Percent (per 10%)	1.04	0.95-1.14	0.42	1.13	1.01-1.26	0.03

Only percentage, not intensity, of tumor cells staining was significantly associated with overall survival. Subgroup analysis by histologic type and neoadjuvant therapy did not show significant associations between percentage or intensity of tumor cells staining and survival.

DISCUSSION

This study showed that higher SULF2 expression by immunohistochemistry is associated with worse overall survival in esophageal cancer. After adjusting for age, sex, race, histologic type, stage, and neoadjuvant therapy, for every 10% increase in percentage of tumor cells staining for SULF2, the risk of death increases by 13%. As expected, higher stage was also significantly associated with worse overall survival. Interestingly, patients who underwent neoadjuvant therapy had worse overall survival than those who did not. This result likely reflects selection bias, in which patients diagnosed with more aggressive tumors are more likely to receive neoadjuvant therapy.

This study also showed that SULF2 staining differed by histologic type, likely reflecting their distinct etiologies. Squamous cell carcinoma samples had significantly higher percentage and intensity of tumor cells staining than adenocarcinoma samples. These results correspond to our previous findings in non-small cell lung cancer, in which in ten of ten squamous cell carcinoma samples showed staining for SULF2, while zero out of ten adenocarcinoma samples showed staining in the tumor cells.[16] However, in the aforementioned study, all squamous cell carcinoma and adenocarcinoma samples showed SULF2 staining of tumor stroma cells.

The association of SULF2 and overall survival corresponds to previous findings in two other types of cancer. Lai et al. showed that increased *SULF2* transcript expression was associated with worse overall survival in patients with hepatocellular carcinoma.[15] Tessema et al. showed that *SULF2* promoter methylation was associated with improved overall survival in patients with non-small cell lung cancer.[17] Our results show that increased SULF2 at the protein level is associated with worse overall survival in a third type of cancer, consistent with an important role for this extracellular enzyme in carcinogenesis.

The *SULFs* were first discovered in a study on quail embryo development, with the human orthologs, *SULF1* and *SULF2*, cloned soon thereafter.[10] SULFs have been shown to regulate several signaling pathways by changing the sulfation status of extracellular heparan sulfate proteoglycans. Ai et al. established that the SULFs promote canonical Wnt signaling, by removing 6OS from the heparan sulfate chains of heparan sulfate proteoglycans, allowing the Wnt ligands to interact with its Frizzled receptor, leading to activation of Wnt target genes.[7]

Originally, SULFs were thought to be tumor suppressors, because forced expression of a SULF in several tumor cell lines caused reduced growth-factor signaling by HB-EGF, FGF-2, or HGF, as well as diminished tumorigenicity.[19-22] Soon, however, they were thought to play an oncogenic role, as one or both SULF genes were found to be overexpressed in subsets of multiple tumors (breast, pancreatic, hepatocellular carcinoma, head and neck, lung, multiple myeloma, and glioblastoma).[10,11,13-16,20,23-25] SULF2 in particular has been identified as a candidate cancer-causing gene in two unbiased screening studies in human breast cancer and mouse brain cancer.[26,27] Moreover, in pancreatic and lung cancer cell lines, SULF2 knockdown led to reduced proliferation and reduced growth of xenografts in mice, likely due to its effect on canonical Wnt signaling pathway.[13,16] In hepatocellular cancer cell lines, overexpression of SULF2 led to increased proliferation and migration and markedly enhanced the tumorigenicity of the cells in nude mice.[15] Similarly, a recent study showed SULF2 transcripts and protein upregulation in human malignant astrocytoma, and, using knockdown and transgenic approaches, demonstrated a SULF2-dependent increase in PDGFR signaling,

tumor cell proliferation in vitro, and tumor growth in vivo.[14] The present study is the first to investigate SULF2 in esophageal cancer.

Our results show that SULF2 was expressed in 93 of 100 human esophageal tumors. Importantly, high expression of SULF2 was associated with worse prognosis in esophageal cancer. As a secreted molecule, SULF2 is a potential diagnostic or prognostic biomarker. Current screening programs for patients with Barrett's esophagus or severe gastroesophageal reflux are ineffective in preventing esophageal adenocarcinoma, due to the slow rate of progression and resultant low incidence of esophageal cancer.[4] The development of SULF2 as a biomarker may help identify a high-risk group that would make screening more feasible. In our study, 15 out of 20 (75%) adjacent Barrett's esophagus samples had SULF2 staining. Also, 5 out of 5 (100%) adjacent carcinoma in situ samples had SULF2 staining, although 3 out of 6 (50%) adjacent benign squamous epithelium had SULF2 staining as well. Future studies are needed to evaluate SULF2 specifically in these precursor lesions as well as in blood or other body fluids.

Given the prevalence and poor prognosis of esophageal cancer, advances in chemotherapy and targeted therapies based on key molecular pathways are greatly needed**.** Although further investigations are required for validation of the role of SULF2 in esophageal cancer, our results raise the possibility that SULF2 could be a therapeutic target. SULF2 is an extracellular enzyme and thus is potentially amenable to inhibition by either antibody-based or small-molecule drugs. *SULF1* and *SULF2* double knockout mice had increased perinatal mortality, and the mice that did survive had lower body weight.[28]; however *SULF1* and *SULF2* single knockout mice appear normal and have normal survival, suggesting that each enzyme might be singly targeted.

In conclusion, we show SULF2 expression in esophageal carcinoma. The vast majority of adenocarcinoma samples and all of the squamous cell carcinoma samples had some degree of SULF2 protein expression. Higher percentage of tumor cells staining for SULF2 is significantly associated with worse overall survival in these patients. Patients with esophageal cancer have an extremely poor prognosis, and SULF2 is a promising biomarker that could play an important role in the diagnosis and prognosis.

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Competing Interests

None

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Contributorship Statement

NSL: study design, data collection, patient selection, statistical analysis and interpretation, paper writing; MVZ: patient selection, reviewing H&E slides, scoring SULF2 stained slides; SDR, DMJ: data analysis and interpretation; HLA: study design, data analysis and interpretation, paper writing.

Data Sharing Statement

No additional data available.

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Figure Legends

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.

Figure 2. Representative sections from adenocarcinoma samples with no staining **(A)**, weak staining **(B)**, and moderate staining **(C)**; and a squamous cell carcinoma sample with strong staining **(D)**. Endothelial cell staining served as an internal positive control, as seen in the upper left corner of **(A)**.

Figure 3. SULF2 staining of adjacent Barrett's esophagus.

Figure 4. Kaplan-Meier survival estimates by **(A)** stage, **(B)** histologic type, **(C)** neoadjuvant therapy, and **(D)** percentage of tumors cells staining. P-values were calculated using the log-rank test.

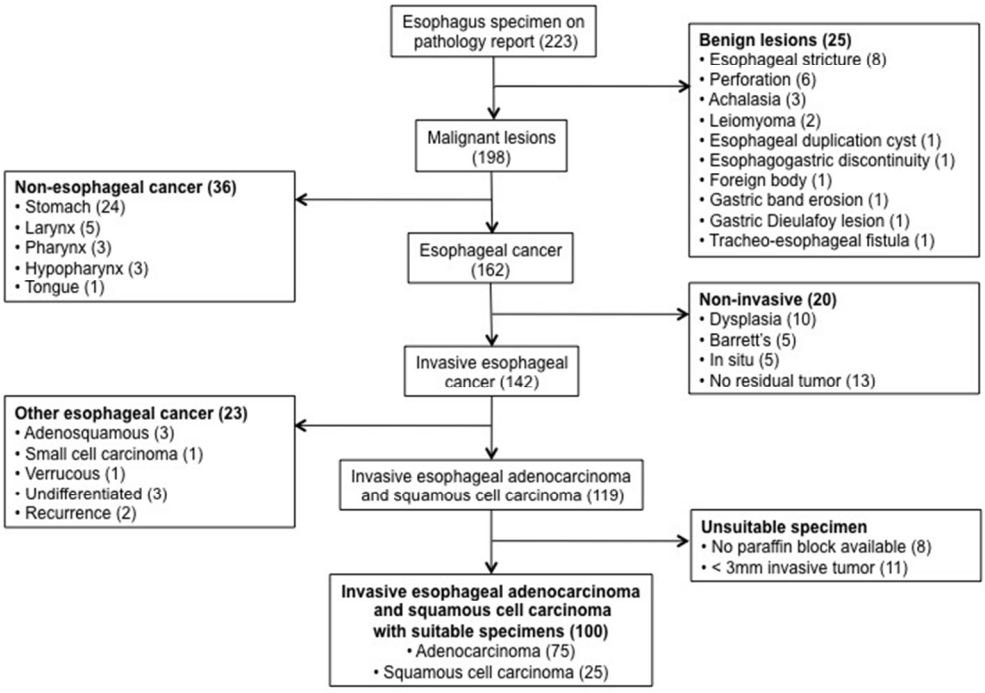

Figure 1. Patient selection by review of 223 patients who underwent esophagectomy from 1997 to 2006.
254x190mm (72 x 72 DPI)

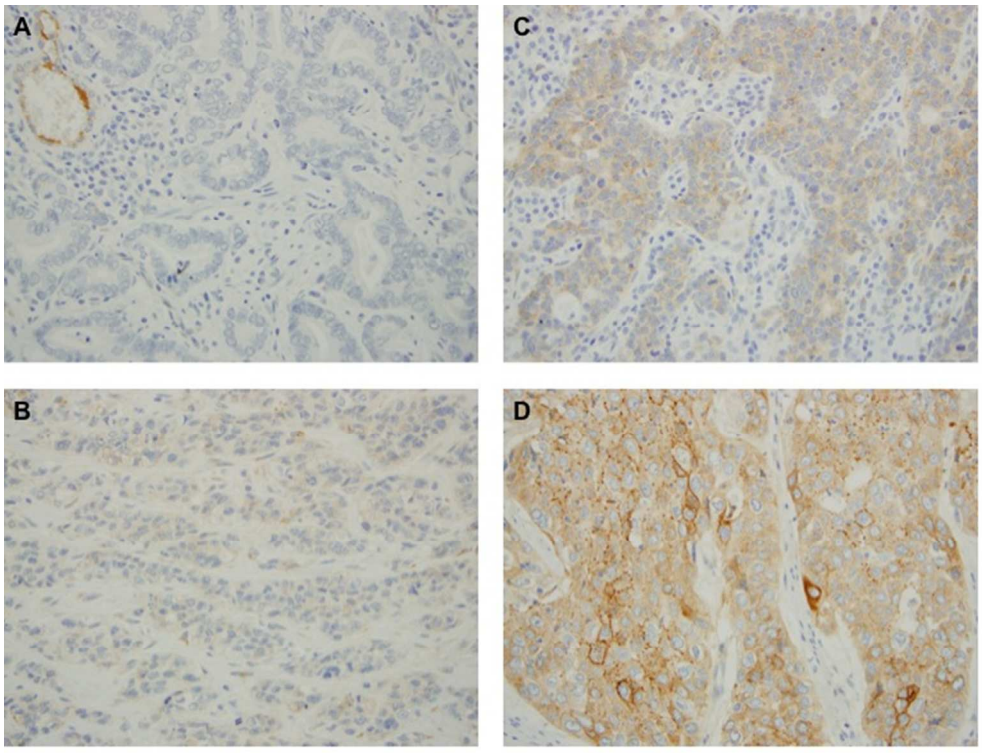

Figure 2. Representative sections from adenocarcinoma samples with no staining (A), weak staining (B), and moderate staining (C); and a squamous cell carcinoma sample with strong staining (D). Endothelial cell staining served as an internal positive control, as seen in the upper left corner of (A).
254x190mm (72 x 72 DPI)

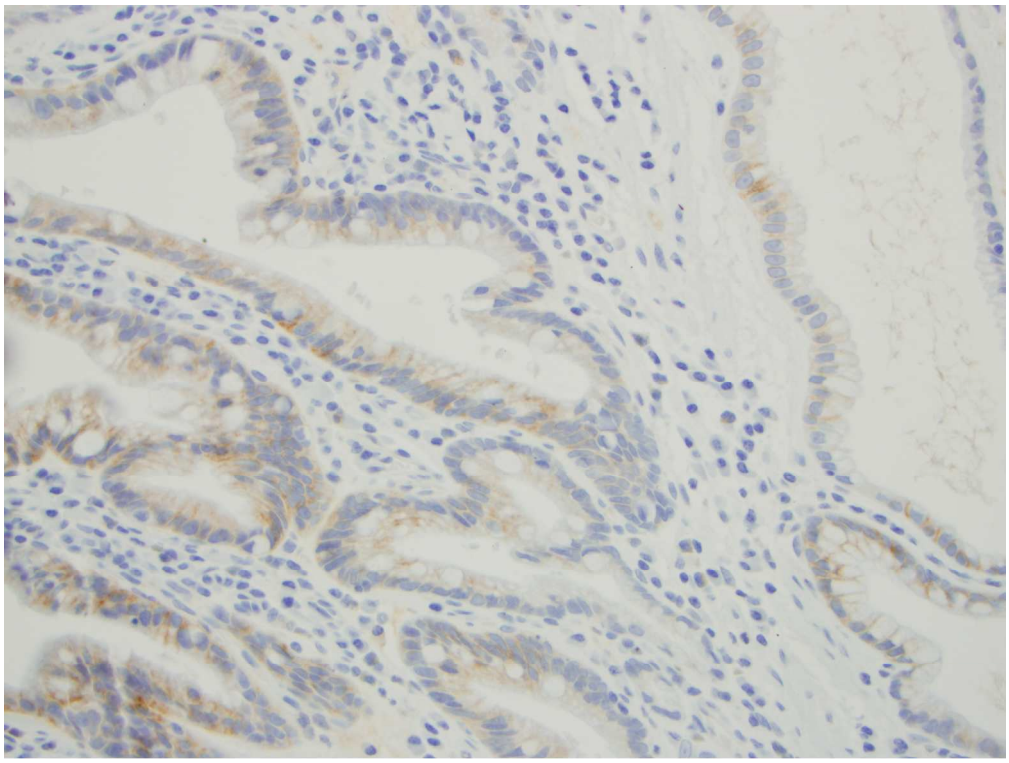

Figure 3. SULF2 staining of adjacent Barrett's esophagus.
239x180mm (216 x 216 DPI)

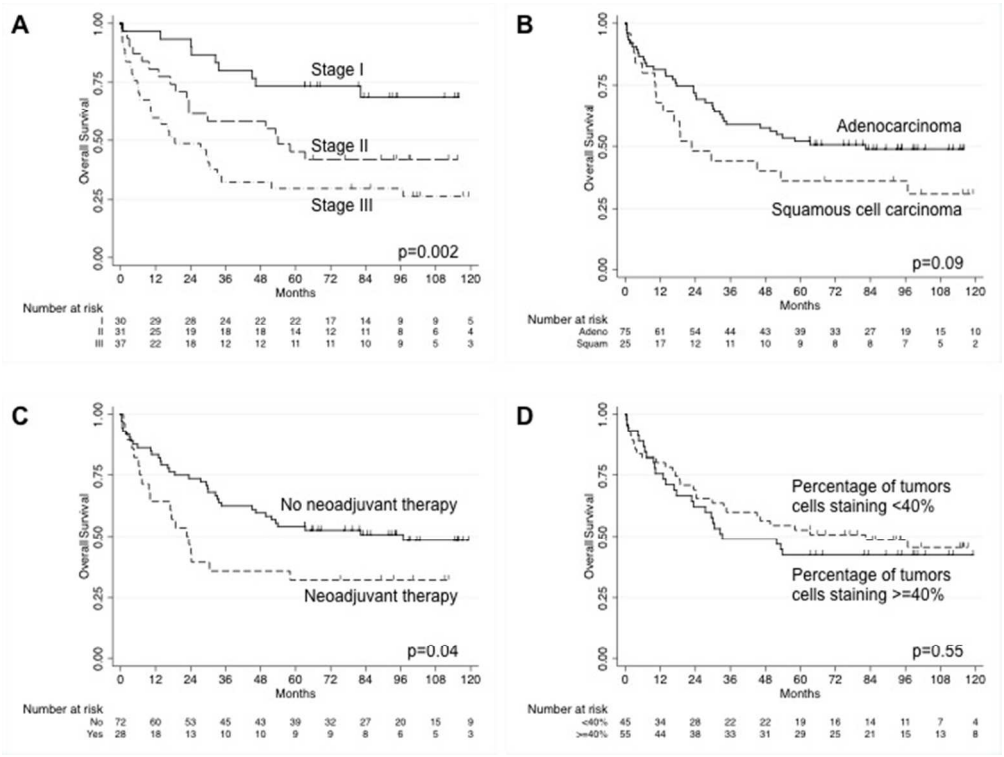

Figure 4. Kaplan-Meier survival estimates by (A) stage, (B) histologic type, (C) neoadjuvant therapy, and (D) percentage of tumors cells staining. P-values were calculated using the log-rank test.
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