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ANATOMICAL PATHOLOGY

Expression of cancer stem cell markers is prognostic in metastatic gastroesophageal adenocarcinoma

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Summary

Gastroesophageal adenocarcinoma is a common and highly lethal malignancy. Cancer stem cells (CSCs) have a key role in the development and progression of metastatic disease. While expression of CSC markers CD44, CD133 and aldehyde dehydrogenase 1 (ALDH1) in locoregional gastroesophageal cancer is known to be associated with poorer clinical outcomes, the significance of CSC marker expression in distal metastatic disease is unknown. We investigated the clinicopathological and prognostic associations of the CSC markers, CD44, CD133, and ALDH1, on metastatic deposits from gastroesophageal adenocarcinomas, and evaluated the association of CSC expression with urokinase-type plasminogen activator receptor (uPAR) expression. Of the 36 patients included in the study, 16 (44%) were positive for CD44, 13 (36%) were positive for CD133, and 26 (72%) were positive for ALDH1. CD44 expression was significantly associated with poorer overall survival (OS) in univariate [hazard ratio (HR) 2.9, 95% confidence interval (CI) 1.3–6.9, p=0.008] and multivariate analyses (HR 2.5, 95%CI 1.1–6.2, p=0.04). ALDH1 expression was significantly associated with poorer OS in univariate (HR 2.4, 95% CI 1.01–5.7, p=0.04) analysis but was not significant in multivariate analysis. Both CD44 and ALDH1 expression were significantly associated with uPAR expression. We found no association between CD133 expression and OS. CD44 expression on metastatic disease from gastroesophageal adenocarcinomas is an independent prognostic marker associated with poorer OS. These results expand current evidence to support the role of CSCs as biomarkers in metastatic gastroesophageal cancer.

Key words: Immunohistochemistry; gastrointestinal neoplasms; cancer stem cell; CD44; CD133; ALDH; uPAR.

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INTRODUCTION

Gastroesophageal and gastric adenocarcinomas (henceforth termed gastroesophageal cancers), are common and lethal malignancies, and a leading cause of cancer mortality worldwide.[1] Despite advances in treatment, prognosis remains poor due to high rates of recurrence after curative surgery, and limited response to systemic treatment in advanced disease.[2] There is an urgent need for novel therapeutic strategies to improve treatments for patients with gastroesophageal cancer.

The cancer stem cell (CSC) hypothesis seeks to explain the high rate of relapse and resistance to current anti-neoplastic treatments. The CSC model proposes that tumour formation, maintenance, and growth is driven by a small population of self-sustaining cells which possess stem cell properties of longevity and infinite proliferation, and are able to differentiate into the wide range of cells forming the heterogeneous tumour mass.[3,4] CSCs, first demonstrated in acute myeloid leukaemia, have been described in most solid tumours including gastroesophageal cancer,[5] breast cancer,[6] prostate cancer,[7] pancreatic cancer,[8,9] melanoma,[10] colon cancer,[11,12] and brain cancer.[13,14]

CSC theory has important clinical implications, as it infers that treatment should be directed to the small pool of CSCs, as well as the large, terminally differentiated tumour bulk. Lineage tracing studies show that CSCs are able to reconstitute the entire tumour bulk following chemotherapy.[15] Promising results from early clinical studies suggest that the inherent resistance of CSCs to chemotherapy and radiotherapy can be overcome by the combination of chemotherapy with CSC targeted treatment in gastric cancer.[16,17]

While numerous proteins have been identified as potential markers of CSC in gastroesophageal cancer including CD24,

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CD49, Sox2, Oct4, and Nanog, the most consistent evidence is for three main markers: CD44, CD133 and aldehyde dehydrogenase 1 (ALDH1).[5]

CD44 is a transmembrane glycoprotein with important roles in matrix adhesion, cell migration, growth, and survival.[18,19] CD44 positive cells from gastric cancer cell lines are shown to be more tumourigenic in mouse and *in vitro* models,[20–22] and resistant to chemotherapy and radiotherapy.[20,22] CD44 is expressed in 44–63% of resected primary gastric cancers,[23,24] and is associated with larger tumour size, depth of invasion, advanced TNM stage, and positive lymph nodes.[24–26] Primary tumour CD44 expression is an independent prognostic factor associated with increased risk of recurrence and poorer overall survival in resected gastric cancer.[25–27]

CD133 is a cell surface transmembrane glycoprotein with a proposed role as an organiser of plasma membrane topology.[28] Preclinical work shows that CD133 positive cells isolated from cell lines demonstrate stem cell properties, and are more resistant to chemotherapy.[13,14,29] Rates of CD133 expression on primary gastric cancer resection specimens range from 25–90%.[30,31] Expression on resected primary gastric tumours is associated with higher risk pathological features, and is an independent factor associated with worse clinical outcomes.[24,30,32–35]

ALDH1 is a member of a family of intracellular enzymes contributing to cellular detoxification, differentiation, and drug resistance.[36] *In vitro*, ALDH1 positive cells from gastric cancer cell lines show self-renewal, heterogeneous linage and increased tumourogenicity.[37] Primary tumour ALDH1 expression is associated with higher TNM stage and pathological factors.[24,38]

The CSC model proposes disseminated CSCs to be the source of metastases, either as primary circulating CSCs or by dedifferentiation though phenotypic plasticity.[39] The expression of CSC markers has been linked to the development of metastatic disease in a variety of malignancies including gastric,[27,34] colorectal,[40] breast[41] and pancreatic[8] cancer.

A key step in the formation of metastatic deposits is invasion of the tumour cells into the surrounding normal tissue. This is facilitated through the urokinase type plasminogen activator (uPAR) system, the critical proteolytic pathway and predominant source of malignant plasminogen activation.[42] Expression of the uPAR system is an important independent prognostic factor for many solid malignancies including gastroesophageal cancer,[43–45] and has an emerging role in CSC signalling.[46,47]

Although the expression of CSCs markers has been well characterised in resected locoregional gastroesophageal adenocarcinoma, there are no data on the expression of CSC markers in metastatic disease. In the current study, we analysed the IHC expression of CD44, CD133 and ALDH1 on metastatic gastroesophageal cancer deposits, and correlated expression with prior treatment, clinicopathological factors, uPAR expression, and clinical outcomes.

MATERIAL AND METHODS

Patient population and tissue samples

We retrospectively identified patients with metastatic gastric or gastroesophageal junction adenocarcinomas treated at two Australian tertiary hospitals (Wollongong Hospital and St George Hospital) who had an available tissue sample from a metastatic site suitable for staining and scoring (n=36). Clinicopathological variables extracted from patient records included: age, sex, tumour histological type, grade, site of metastases, Eastern Cooperative Oncology Group (ECOG) performance status, treatments, progression free survival (defined as the time from the date of primary treatment to the date of progression or death) and overall survival (time from diagnosis to death from any cause). The study was approved by South Western Sydney Local Health District Human Research Ethics Committee (Project Number 15/072).

Immunohistochemistry

We used formalin fixed, paraffin embedded tissues from patients who underwent biopsy of a metastatic deposit from primary gastroesophageal adenocarcinoma. Immunohistochemical staining was carried out as previously reported.[24] Freshly cut 4 µm sections from patient tissue blocks were mounted on aminopropylethoxysilane precoated glass slides. Sections were deparaffinised in EZ Prep and washed in Reaction Buffer (Ventana Medical Systems, USA). The immunohistochemical staining was performed using defined protocols with the Ventana BenchMark Ultra Automated IHC/ISH slide staining system. Antigen retrieval was performed by incubation at 100°C at pH 9.0 for between 24 and 32 min. Incubation with primary antibodies was carried out at 37°C for one hour. Sections were incubated with the following antibody dilutions: anti-CD44 (1:200; clone EPR1013Y; Abcam, UK), CD133 (1:100; clone AC133; Miltenyi Biotec, Germany), ALDH1 (1:100; clone 44; BD Transduction Laboratories, USA), and uPAR (1:100; clone R4; Dako, Denmark). A post primary endogenous peroxidase inhibition was performed by incubating the slides in 1% hydrogen peroxide for 15 min. Development of colour was achieved by 15 min incubation with diaminobenzidine (DAB) solution, followed by counterstaining with haematoxylin. Sections from normal human epidermis, colon adenocarcinoma and normal human appendix were used as positive controls for CD44, CD133 and ALDH1, respectively.[24,36] All staining runs were accompanied by appropriate control slides.

Scoring of immunohistochemical staining

CSC scoring was performed by two independent pathologists blinded to clinical details (AL, and AI or NH). Previous reports have shown a significant correlation between CD44, CD133 and ALDH1 expression and prognosis in primary gastroesophageal cancer.[5] To remain consistent with the literature,[24,27,48] CD44 and CD133 staining was considered positive if at least 10% of the tumour cells were stained. We noted a much higher proportion of ADLH1 positive cases than previous studies (only 5 cases were negative using a 10% cutoff); accordingly, we increased the threshold to 20% (positive result if 20% or more of the tumour cells stained). Staining in surrounding stroma was not included in the score for any CSC marker.

uPAR scoring was performed by a third blinded pathologist (MI) experienced with immunohistochemical analyses of the uPA system in cancer.[43,49] uPAR expression on cancer cells varies between the tumour core, and the invading edge of the tumour.[49] Analysis of uPAR expression was restricted to cancer cells at the peripheral invasion zone as this has been shown to be prognostic in gastric cancer.[44] Neutrophils were used as internal positive controls on each slide. Scoring was performed as previously reported for gastroesophageal cancer: 0, no uPAR positive cells; 1, less than 1% uPAR positive cells; 2, 1–5% uPAR positive cells; 3, 5–10% uPAR positive cells; 4, more than 10% uPAR positive cells. Samples were considered to be uPAR positive if >5% of tumour cells were stained.[43,44]

Statistical analysis

The primary endpoint of this analysis was overall survival (OS) by CSC expression. Summary statistics of patients' demographic and clinicopathological details, and staining status were provided in frequencies and percentages. Bivariate correlations between clinicopathological features and CD44, CD133, and ALDH1 expression were performed using the Fisher's exact test. A Cox proportional hazard model was used to estimate effects of CD44, CD133 and ALDH1 positivity on each survival endpoint; only covariates significant in univariate analysis were included in the multivariate model. uPAR expression was not included in the multivariate model as staining was only available for a subset of patients. All statistical analyses were performed using SAS 9.2 software (SAS Institute, USA).

RESULTS

Patient characteristics and correlation with CSC expression

Characteristics of the 36 patients are summarised in Table 1. Median follow-up was 5.2 months (interquartile range 2.8–10.7 months). Consistent with the poor prognosis of this disease, most patients (n=32, 89%) had died of their disease. Seventeen (45%) patients received treatment for loco-regional disease prior to developing metastases, although in all cases this was more than 6 months prior to biopsy. Most patients (n=32, 89%) received treatment for the metastatic gastroesophageal cancer including chemotherapy (usually a platinum, fluoropyrimidine and anthracycline combination), radiotherapy, or surgery (Table 1). Radiotherapy and surgery were employed as palliative local treatments for symptomatic metastases.

Table 1 Characteristics of included patients

Characteristic	*n* (%)
Age, median (range)	64 (39–78)
Sex	
Male	29 (80)
Female	7 (19)
Primary tumour location	
GOJ	16 (44)
Gastric body	20 (56)
Site of metastatic biopsy	
Pulmonary	4 (11)
Peritoneum/omentum/ascites	18 (50)
Liver	7 (18)
Bone	2 (5)
Distal lymph node[a]	4 (11)
Soft tissue	2 (5)
ECOG performance status	
0–1	31 (86)
2–4	5 (14)
Prior treatment[b]	
Surgery	13 (34)
Chemotherapy	13 (34)
Radiotherapy	5 (13)
Nil	21 (55)
Treatment for metastatic disease	
Chemotherapy	26 (68)
Immunotherapy	1 (3)
Radiotherapy	7 (18)
Surgery	3 (8)
Nil	6 (16)

GOJ, gastroesophageal junction.
[a] Site of distal lymph nodes include mediastinal, supraclavicular, and para-aortic.
[b] All prior treatment was curative intent.

Of all cases, 16 (44%) were positive for CD44, 13 (36%) were positive for CD133, and 26 (72%) were positive for ALDH1 (Fig. 1). We found no association between CSC markers and clinicopathological features, including primary tumour location, site of metastatic disease or biopsy sample, previous chemotherapy exposure, or histopathology (Table 2).

Correlation of CSC marker and uPAR expression

Samples including the peripheral invasion zone were available for 28 samples (8 samples excluded, due to insufficient tissue n=4, or the biopsy included tumour core only n=4). Nine of 28 (32%) samples were positive for cancer cell uPAR (Fig. 2). CD44 and ALDH1 expression was significantly associated with tumour cell uPAR expression (p=0.02 and 0.03, respectively, Table 3), with higher tumour uPAR expression in CD44 and ALDH1 positive cases. There was no association between CD133 and uPAR expression.

CSC marker expression and prognosis

In univariate analysis, CD44 positive cases had a poorer OS than CD44 negative cases [hazard ratio (HR) 2.9, 95% confidence interval (CI) 1.3–6.9, p=0.008, Table 4]. Similarly, ALDH1 positive cases had a poorer OS than ALDH1 negative cases (HR 2.4, 95%CI 1.1–5.7, p=0.04). There was no

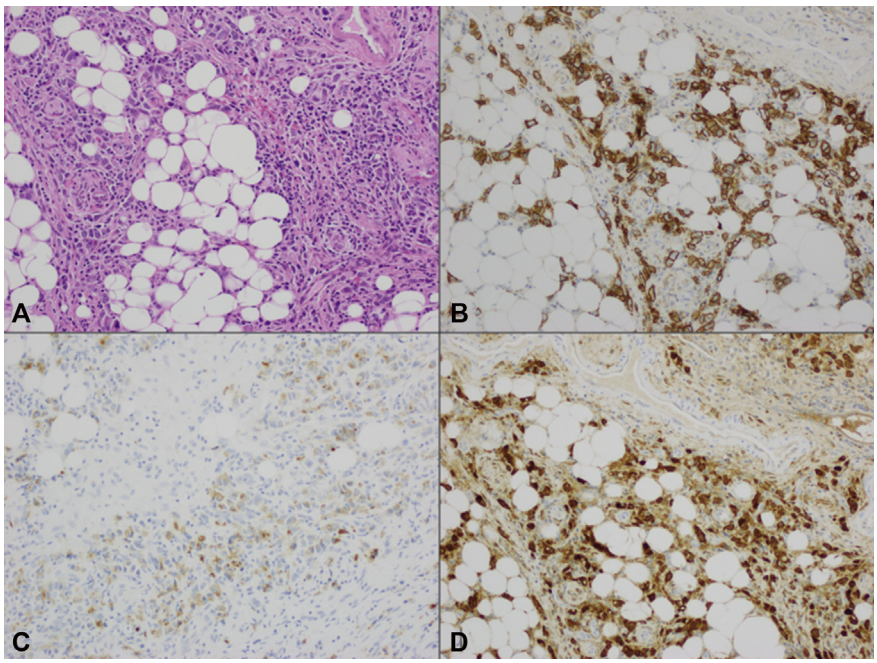

Fig. 1 Representative staining of CSC markers on metastatic deposits with corresponding haematoxylin and eosin (H&E) stain. (A) H&E stain, (B) membranous staining of CD44, (C) CD133 staining in apical membranes, (D) cytoplasmic staining of ALDH1.

Table 2 Association of CSC marker staining with clinicopathological features (n=36)

	CD44		CD133		ALDH1	
Total (%) % positive cells (mean)	16/36 (44%) 10–100% (62)		13/36 (36%) 10–100% (38)		26/36 (72%) 10–100% (76)	
	Positive rate	p value	Positive rate	p value	Positive rate	p value
Primary location						
GOJ	8/16	0.73	6/16	0.98	12/16	0.98
Gastric	8/20		7/20		14/20	
Sites of metastatic disease						
Peritoneal/omentum only	7/18	0.73	4/18	0.16	14/18	0.71
Other	9/18		9/18		12/18	
Previous chemotherapy						
Yes	6/13	0.87	5/13	0.83	10/13	0.72
None	10/23		8/23		16/23	
Histopathology						
Well/moderately differentiated	5/16	0.19	5/16	0.73	5/16	0.72
Poorly differentiated	11/20		8/20		15/20	

CSC, cancer stem cell; GOJ, gastroesophageal junction.

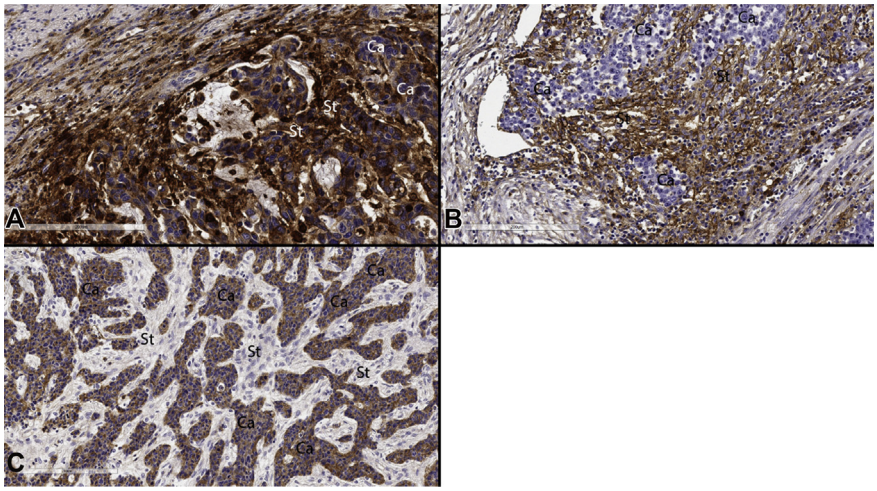

Fig. 2 Representative images of three patterns of uPAR staining in metastatic gastroesophageal samples. (A) There is widespread strong uPAR expression on cancer cells (Ca) and macrophages and myofibroblasts (stromal cells, St). (B) Strong stromal but weak cancer cell staining. (C) Weak stromal staining with strong cancer cell expression. Only cancer cell expression was quantified and included in the current study.

significant difference in OS between CD133 positive and negative cases (HR 1.16, 95%CI 0.57–2.4, p=0.67) (Fig. 3).

In multivariate analysis, after adjusting for performance status, tumour grade, and treatment, CD44 positivity remained a significant independent predictor of OS (HR 2.5, 95%CI 1.1–6.2, p=0.04), while ALDH1 became non-significant (HR 2.0, 95%CI 0.86–5.1, p=0.1) (Table 4).

Expression of combinations of CSC markers was also examined for association with OS. Patients with CD44+/ALDH1+ expression (14/36, 39%) had a significantly poorer OS in univariate (HR 4.1, 95%CI 1.7–9.5, p=0.0006) and multivariate analysis (HR 4.0, 95%CI 1.6–10.1, p=0.002). No combination including CD133 was significantly associated with OS.

Table 3 Association of CSC marker staining with uPAR staining (n=28)

	Positive uPAR staining	p value
CD44		
Positive	7/15 (54%)	0.02
Negative	2/13 (13%)	
CD133		
Positive	1/9 (11%)	0.10
Negative	8/19 (42%)	
ALDH1		
Positive	9/12 (43%)	0.03
Negative	0/7 (0%)	

CSC, cancer stem cell; uPAR, urokinase-type plasminogen activation receptor.

DISCUSSION

In the CSC model, establishment and progression of metastatic disease is due to the dissemination of CSCs. While numerous previous studies have demonstrated expression of CSC markers in loco-regional gastroesophageal cancer to be significantly associated with clinical outcomes, the current study is the first to examine the expression of CSC markers in metastatic gastroesophageal cancer.

We found expression of CD44 and ALDH1, but not CD133, on metastatic deposits to be significantly associated with poorer OS. In multivariate analysis, after adjusting for tumour grade, ECOG performance status, and treatment

Table 4 Univariate analysis and multivariate analysis

Characteristic	Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*
ECOG performance status				
0-1	**1**	**0.0014**	1	0.75
2-4	**7.3 (2.1–25.0)**		1.2 (0.4–6.2)	
Age				
<65	1	0.79		
≥65	1.1 (0.5–2.2)			
Sex				
Female	1	0.42		
Male	1.5 (0.56–3.9)			
Primary location				
GOJ	1	0.09		
Gastric	0.55 (0.26–1.1)			
Site of metastatic disease				
Peritoneal/omentum only	1	0.16		
All other sites	0.60 (0.29–1.2)			
Histopathology				
Well/mod differentiated	**1**	**0.0003**	**1**	**0.007**
Poorly differentiated	**3.9 (1.8–8.6)**		**3.3 (1.4–7.9)**	
Treatment				
None	**1**	**0.001**	**1**	**0.03**
Systemic treatment	**0.27 (0.12–0.62)**		**0.28 (0.1–0.88)**	
Radiotherapy	1.1 (0.5–2.9)	0.71[a]		
Surgery	2.2 (0.6–7.5)	0.19[a]		
CD44				
Negative	**1**	**0.008**	**1**	**0.04**
Positive	**2.9 (1.3–6.9)**		**2.5 (1.1–6.2)**	
CD133				
Negative	1	0.67		
Positive	1.16 (0.57–2.4)			
ALDH1				
Negative	**1**	**0.04**	1	0.1
Positive	**2.4 (1.01–5.7)**		2.0 (0.86–5.1)	

Values in bold are significant.
CI, confidence interval; ECOG, Eastern Cooperative Oncology group; GOJ, gastroesophageal junction; HR, hazard ratio.
[a] Radiotherapy and surgery were given as palliative local treatments only, and therefore had no impact on survival and were not incorporated into the multivariate model. Systemic treatment included chemotherapy or immunotherapy.

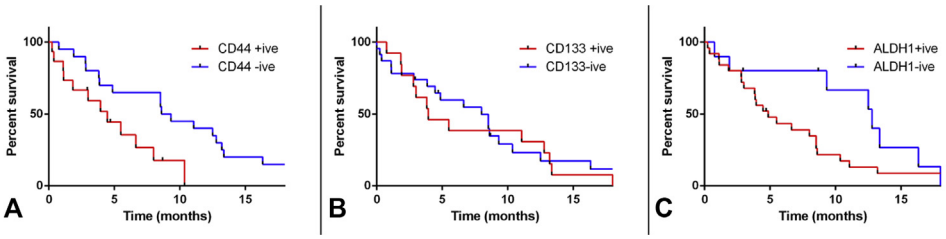

Fig. 3 Kaplan–Meier survival curves for overall survival stratified by cancer stem cell marker expression. (A) CD44: univariate HR 2.9, 95%CI 1.3–6.9, p=0.008. (B) CD133: univariate HR 1.16, 95%CI 0.57–2.4, p=0.67. (C) ALDH1: univariate HR 2.4, 95%CI 1.1–5.7, p=0.04.

received, CD44 expression remained a significant prognostic factor associated with poorer OS (HR 2.5, 95%CI 1.1–6.2, p=0.04). ALDH1 expression was not significantly associated with OS in multivariate analysis (HR 2.0, 95%CI 0.86–5.1, p=0.1), although the combination of CD44+/ALDH1+ was strongly associated with poorer OS (HR 4.0, 95%CI 1.6–10.1, p=0.002). This finding confirms previous work showing the importance of CSC expression, particularly CD44, as a biomarker in gastroesophageal cancer.[24,27,34] Our results did not show any association between CD133 with OS, either alone or in combination with CD44 or ALDH1. Recent work suggests that only a subset of CD133 positive CSC are essential for tumour metastases.[8] We hypothesise that additional markers, such as CXCR4, are required in combination with CD133 to identify this key subgroup.

We also found expression of CD44 and ALDH1 to be significantly associated with expression of uPAR. Our results mirror previous work in other solid tumours showing co-expression of uPAR with CSC markers.[50–52] In addition to a well characterised role in the uPA system, there is increasing evidence suggesting uPAR has an important function in CSCs. uPAR overexpression is strongly correlated with the CSC properties of an invasive phenotype, drug resistance, and poor prognosis.[53] Moreover, signalling by uPAR induces stem cell-like properties in breast, brain, lung and prostate cancer cells.[46,47,54–56] To the best of our

knowledge, this study is the first to show the co-expression of CSC and uPAR in gastroesophageal cancers, and further supports the role of uPAR in CSCs.

In resected loco-regional gastroesophageal cancer, the proportion of CD44 and CD133 positive cases is estimated at 17–77% and 10–44%, respectively.[57] Our results demonstrated a similar proportion of CD44 positive (44%) and CD133 positive (36%) cases, but a higher expression of ALHD1 than that seen in loco-regional disease (73% positive cases compared to 50–55%).[24,38] This is despite using a higher cut-off for positive cases (20% of cells stained compared to 10%). In addition, most ALDH1 cases were diffusely and strongly stained (mean proportion of positive cells 76%). Our results support a previously identified trend of higher ALDH1 expression on local nodal deposits. In a study comparing IHC expression of CSC between primary gastric cancers and matched lymph node metastases, Wakamatsu *et al.* also found a higher expression of ALDH1, but not CD44 or CD133, in the lymph nodes.[24] ALHD1 expression is strongly correlated with expression of matrix metalloproteases (MMPs), which are essential for extracellular matrix degradation and establishment of metastatic disease.[38]

We were unable to show any significant association between CSC staining and other important clinicopathological factors. This is in contrast to other IHC studies which have shown strong associations between poor pathological factors, such as TNM stage, tumour invasion and grade with expression of CSCs.[5] The small sample size of our study is likely to be a contributing factor. It is interesting to note we did not find a higher CSC expression in patients with previous chemotherapy exposure. While CSCs are known to be relatively chemotherapy insensitive, leading to enrichment of CSCs with chemotherapy, modern CSC models describe a dynamic CSC population with a bidirectional pathway between CSC and differentiated cell populations.[58] As no patient had received chemotherapy within 6 months prior to the biopsy, it is likely that the CSC population had re-established an equilibrium with the terminally differentiated tumour bulk.

The key role of CSCs in metastatic gastroesophageal cancer is supported by early clinical results of agents targeting the CSC pathway. For example, in a phase II study using the hedgehog inhibitor vismodegib with chemotherapy in metastatic gastric cancer, Yoon *et al.* found a survival benefit restricted to patients who had a high expression of CD44.[16] Even more novel approaches using the combination of immunotherapy and CSCs are under investigation, with currently recruiting clinical trials employing immune targeting of CSC using dendritic cells.[59] The coexpression of CSC markers and uPAR may provide additional opportunities to target CSC using uPAR directed therapies.[52]

It is important to acknowledge several limitations of this study. Firstly, we found that most gastroesophageal cancers, even when metastatic, have histological diagnosis on endoscopy and biopsy, rather than biopsy of metastatic deposits. This limited the available patient population and study size for the current work. Secondly, most samples used in the current study were biopsy specimens, rather than larger resection specimens, which did not allow exploration of tumour heterogeneity and differential expression of CSC makers. Thirdly, due to technical limitations, uPAR staining was available for most, but not all patients, limiting incorporation into the multivariate OS analysis.

CONCLUSION

Expression of the CSC marker CD44 is an independent prognostic factor associated with poorer OS in metastatic gastroesophageal cancer. This study provides further evidence that expression of CSC markers is a valid biomarker in gastroesophageal cancer, and highlights importance of CSCs in all stages of gastroesophageal cancer.

Conflicts of interest and sources of funding: The authors state that there are no conflicts of interest to disclose.

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