

Gastric cancer stem cells: evidence, potential markers, and clinical implications

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Received: 23 July 2015 / Accepted: 13 September 2015 / Published online: 1 October 2015
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Abstract Gastric cancer is a significant global health problem. It is the fifth most common cancer and third leading cause of cancer-related death worldwide (Torre et al. in *CA Cancer J Clin* 65(2):87–108, 2015). Despite advances in treatment, overall prognosis remains poor, due to tumour relapse and metastasis. There is an urgent need for novel therapeutic approaches to improve clinical outcomes in gastric cancer. The cancer stem cell (CSC) model has been proposed to explain the high rate of relapse and subsequent resistance of cancer to current systemic treatments (Vermeulen et al. in *Lancet Oncol* 13(2):e83–e89, 2012). CSCs have been identified in many solid malignancies, including gastric cancer, and have significant

clinical implications, as targeting the CSC population may be essential in preventing the recurrence and spread of a tumour (Dewi et al. in *J Gastroenterol* 46(10):1145–1157, 2011). This review seeks to summarise the current evidence for CSC in gastric cancer, with an emphasis on candidate CSC markers, clinical implications, and potential therapeutic approaches.

Keywords Stomach neoplasms · Gastric cancer · Cancer stem cells · CD44 · CD44v · CD133 · ALDH1

Cancer stem cells

Cancer stem cell theory

A key issue in oncology is whether cancer growth is driven by the majority of tumour cells or by a rare subpopulation of cancer stem cells (CSCs). There are several proposed models. The clonal evolution model states that each cell within a tumour has equal potential of undergoing genetic/epigenetic changes which confer growth advantages and subsequent new tumour growth [4]. The cancer stem cell (CSC) model, on the other hand, proposes that the growth of the tumour is driven by a small population of self-sustaining cells with the stem cell properties of longevity, infinite proliferation, and an ability to differentiate into the entire heterogeneous population of the tumour [5]. Integral to the CSC model is a subpopulation at the apex of the hierarchy (usually comprising <5 % of the cancer), responsible for the formation, maintenance, and continued growth of the tumour [3]. Stem cells can symmetrically divide into identical daughter stem cells with self-renewal capacity, or asymmetrically divide into both a differentiated progenitor cell and a stem cell [6].

Electronic supplementary material The online version of this article (doi:10.1007/s00535-015-1125-5) contains supplementary material, which is available to authorized users.

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Recent refinements to the CSC theory propose a more dynamic model, with a fluid CSC population regulated by the tumour cell environment rather than a rigid hierarchical structure [2, 5]. It is now apparent that a CSC phenotype can be induced in differentiated cancer cells by exposure to growth factors secreted from stromal cells, suggesting a bidirectional pathway between the CSC and differentiated cell populations [7, 8]. Furthermore, research has shown that some types of leukaemic stem cells are subjected to clonal evolution [9]. The reacquisition of self-renewal properties in non-CSC populations, in addition to the genotypic and phenotypic heterogeneity within CSCs, highlights the fluidity of the CSC population in both number and character (Fig. 1).

CSCs are defined functionally rather than by cellular origin, with CSCs having superior tumour initiation, growth, and metastatic potential to that of other tumour

cells [10]. In vitro studies with cultured gastric CSCs have found these cells to be more resistant to chemotherapy and radiotherapy [11, 12], possibly due to high expression of anti-apoptotic proteins, increased efficiency of DNA repair, and alterations in cell cycle kinetics [5, 6]. CSCs are responsible for the renewal of tumour mass following systemic treatment and the development of treatment-resistant subclones [6]. The long proliferative lifespan and repeated DNA replication events in CSCs render them more susceptible to further mutation and epigenetic changes, creating additional malignant clones [13].

The first definitive evidence of a CSC population was provided by Bonnet et al. [14], who demonstrated that a cell population defined by the CD34+/38– phenotype was able to serially reproduce acute myeloid leukaemia (AML) in immunodeficient mice. Importantly, this cell population was not only capable of self-renewal, but could reconstitute

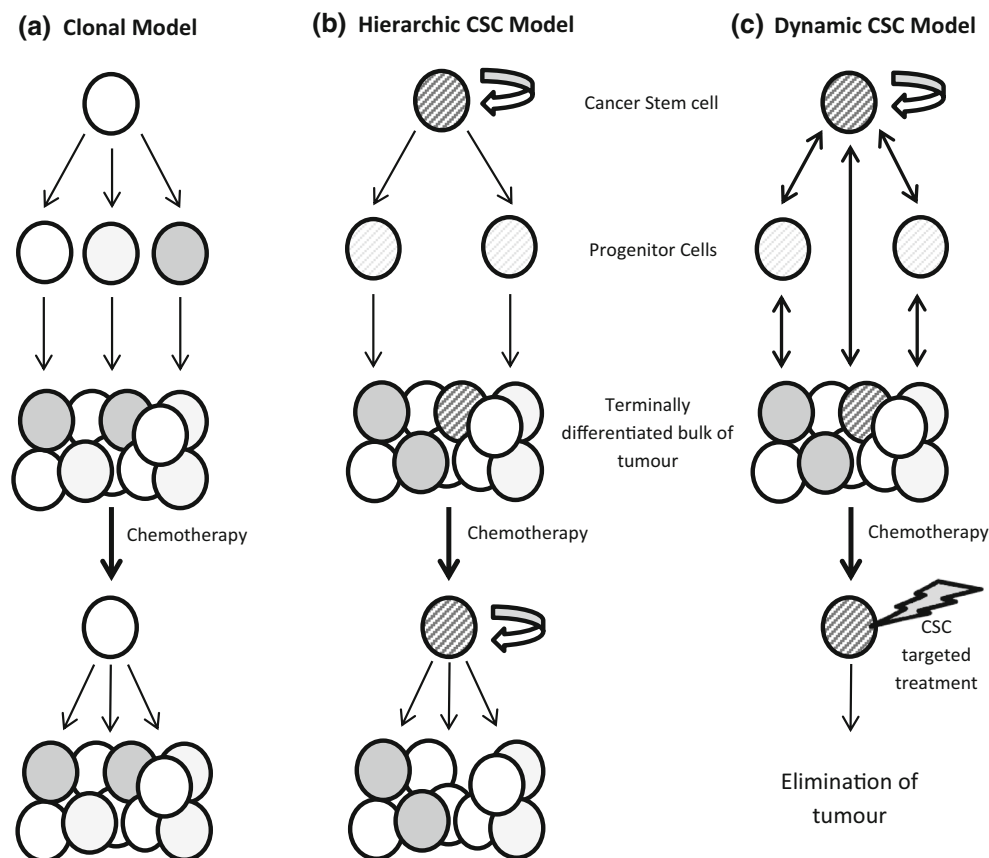


Fig. 1 Three models of tumour growth and their clinical implications. **a** The clonal model, where each cell has the potential to acquire additional mutations which confer a growth advantage. Chemotherapy selects a treatment-resistant subclone, which subsequently reconstitutes the tumour mass. **b** The hierarchical cancer stem cell (CSC) model, whereby a small population of self-renewing CSCs are responsible for all tumour growth, giving rise to progenitor cells, which subsequently de-differentiate into the bulk of the tumour which has lost the capacity for self-renewal. The chemoresistant CSC

population is enriched by chemotherapy, and is able to restore the tumour bulk. **c** The dynamic CSC model is a more refined model, demonstrating the bidirectional flow of cells between the stem cell and differentiated compartments. This model highlights the need to combine a CSC-targeted agent with chemotherapy. The targeted agent eliminates the chemoresistant CSC population, preventing the reconstitution of the tumour bulk, while the chemotherapy reduces the tumour bulk of differentiated cells, stopping these cells from de-differentiating to replenish the CSC population

the full spectrum of cell populations seen in AML. This approach has been used to isolate CSCs in solid malignancies including breast cancer [15], prostate cancer [16], pancreatic cancer [17, 18], melanoma [19], colon cancer [20, 21], brain cancer [22, 23], and liver cancer [24], supporting the model of cancer growth initiated and maintained by CSCs. The presence of CSCs in solid malignancies has been confirmed in lineage tracing studies, which identified a subpopulation of cells that reconstitute the entire tumour following chemotherapy [25].

Experimental evidence for CSCs must demonstrate both self-renewal and an ability to differentiate into the heterogeneous cell populations that constitute a tumour [26]. Serial transplantation in animal models fulfils these criteria, and has been proposed as the best functional assay for identification of CSCs [26]. An alternative experimental model is *in vitro* spheroid colony formation of candidate CSCs in culture media, as continued growth of colonies indicates self-renewal [27]. Although serial passage in animal models is considered the gold standard, the two methods seem to provide similar results in identifying candidate CSCs [27].

The CSC model has important clinical implications, as it implies that anti-neoplastic treatments should focus on eliminating both a small population of CSCs within the tumour and the rapidly dividing but terminally differentiated bulk cancer cells [26]. Figure 1 provides schematic illustrations of the various models.

Cancer stem cells and metastases

The unique CSC properties of self-renewal and multi-lineage differentiation suggest a likely role in the initiation and progression of distant metastatic disease. Although there is no direct experimental evidence of CSCs as the origin of metastases, many studies have provided supporting data [28]. Various works have shown that the presence of unique tumour subpopulations with CSC markers is integral to the development of metastatic disease in a number of malignancies, including pancreatic [17], colorectal [29], and breast [30] cancer, as depletion of this population prevented the metastatic spread of the tumour [17]. Dieter et al. [31] demonstrated differential contributions of individual CSC clones to the growth of primary and metastatic tumours, and identified a subpopulation of CSCs in colon cancer solely responsible for the formation of metastases. In addition, immunohistochemical studies in gastric cancer have shown an increased risk of metastatic disease associated with CSC marker expression in the primary tumour [32, 33].

A proposed mechanism underlying the metastatic progression of cancer is the epithelial-mesenchymal transition (EMT) [34], which is the process whereby tumour cells

lose epithelial characteristics and acquire a mesenchymal phenotype to facilitate cancer metastasis and survival. It is becoming increasingly evident that EMT and CSC phenotypes are largely overlapping, providing properties of invasion, tumour seeding, drug resistance, and survival. CSCs in primary tumours are thought to metastasize to distant sites via EMT [35]. Furthermore, a CSC phenotype can be obtained through induction of an EMT state [36, 37]. Tumour cells disseminated in the blood (circulating tumour cells) are enriched for both EMT and CSC phenotypes [38]. Gastric cancer patients who have detectable circulating tumour cells (CTCs) that express CSC markers have a poorer prognosis than those who have CTCs without CSC markers [39]. This is reinforced by clinical evidence of an association between EMT and CSCs, with immunohistochemical expression of CD44, a gastric CSC marker, significantly correlated with expression of EMT markers such as Snail-1 and ZEB1 in resected gastric cancer [40]. Gastric CSCs isolated from a cell line have shown increased expression of EMT markers (including Snail, Twist, and vimentin) and CD44 [41]. Moreover, analysis of the combined expression of CD44 and EMT markers was predictive of poorer disease-free and overall survival (OS) in a multivariate model, consistent with the aggressive phenotype of cells expressing CSC and EMT markers [40].

Identification of cancer stem cells

A key issue in the study of CSCs is the ability to develop reproducible and reliable methods for CSC isolation and identification. The American Association for Cancer Research (AACR) defines CSCs as subpopulations of cells within a tumour that possess the capacity for self-renewal and generation of heterogeneous lineages of cancer cells that constitute the tumour [26]. As discussed above, two experimental methods are used to confirm a population of cells as CSCs: serial passage of tumours in animal models and tumour-spheroid assays. Animal model serial transplantation is regarded as the gold standard, as it is considered a physiologically relevant functional assay for demonstrating self-renewal and lineage capacity [26]. Some groups have questioned this paradigm, however, as the rarity of CSCs found in human cancers may be the result of an inadequate local environment in the xenograft [42, 43]. Tumour spheroid assays are a more rapid method (as serial transplantation can take several months), and have been shown to increase expression of stem cell markers, although there are potential pitfalls, as not all isolated CSCs form spheroids [12, 44].

There are many challenges in identifying a CSC population within a tumour. First, as discussed above, the CSC population is dynamic, with bidirectional flow between the

CSC and differentiated cell populations. Second, the CSC markers currently in use are not specific, and are expressed on both non-malignant cells and early progenitor cells that have lost stem cell features but retained phenotypic markers. Third, multiple populations of CSCs may exist within a tumour mass, and combinations of multiple markers may be required to identify the complete CSC population.

Candidate CSCs are identified predominantly by one of two methods: the side population assay or the expression of CSC surface markers.

Side population assay

The side population (SP) assay identifies the fraction of cells that efflux Hoechst dye through ATP binding cassette (ABC) transporters. It is a highly preserved marker of stem cells across a variety of tissues and tumours, with a higher capacity for self-renewal, leading some authors to suggest that the SP subset may represent a universal CSC population [45]. However, the SP assay is hampered by poor specificity, with differentiated adult cells in the gastrointestinal tract demonstrating a SP phenotype [46].

The results regarding SP assays as a potential CSC marker in gastric cancer are inconsistent. Zhang et al. [47] showed CSC properties in SP cells from the MKN-45 cell line but not the BGC-823 cell line. Although some studies in gastric cancer lines have shown CSC properties in the SP [48, 49], others have found no difference compared to a non-SP subset [11, 50, 51]. Overall, therefore, the utility of the SP assay for identification of gastric CSCs remains controversial.

Expression of cell surface markers

An integral tool in the identification and isolation of candidate CSCs is the expression of unique combinations of cell surface markers. This approach has allowed the isolation of CSCs in many solid malignancies using flow cytometry or magnetic cell sorting. CSC markers identified to date are expressed in an overlapping manner on a variety of tumours, as well as on normal stem cells. A summary of

the most common markers described for solid tumours is provided in Table 1. Many of these potential markers are found in gastric cancer.

Gastric cancer stem cells

Origin of gastric cancer stem cells

The origin of gastric CSCs remains uncertain. One possible source is gastric stem cells which have lost regulated quiescence. The existence of multipotent gastric stem cells that give rise to all major epithelial cell types has been demonstrated in mouse models [52]. There are multiple populations of gastric stem cells. The Lgr5+ cells arise at the base of the gastric gland and continuously differentiate into all antral unit cells, while the villin+ cells are located at the isthmus and are a quiescent stem cell population which require cytokine stimulation for activation, serving as a stem cell source if the Lgr5+ cells are damaged [53–55]. Sox2+ cells, present in the antrum and corpus, are able to differentiate into all cell types found in a gastric unit, and ablation of the Sox2+ population prevents renewal of gastric epithelium [56]. Other populations of differentiated gastric cells, such as chief cells expressing the marker Troy, have been shown to de-differentiate and function as multipotent stem cells, acting as reserve stem cell populations [57].

Aberrant genetic and epigenetic mutations in these gastric stem cells, in conjunction with stimulating factors from the microenvironment, may lead to the formation of CSCs [13]. In one study, for example, APC deletion in Lgr5+ stem cells in a mouse model led to the rapid development of adenomas, due to the expansion of the stem cell compartment [54]. Wu et al. [53] demonstrated co-localisation of CSC markers (CD26, CD44, ALDH1, CD133) with Lgr5+ cells in gastric cancer, suggesting they may be functionally related. Similarly, Sox2 expression was found to be altered during the pathogenesis of gastric cancer [58, 59], although reports are contradictory as to whether Sox2 is over-expressed and oncogenic [60, 61] or down-regulated and anti-oncogenic, with lower Sox2 levels

Table 1 Cell surface expression of cancer stem cell markers in different solid tumour types

Tumour	Described cell markers	References
Colon	CD133, CD44, CD166, EpCAM, CD24, ALDH1	Botchkina [132]
Pancreas	CD133, CD44/CD24, ALDH1	Zhan et al. [133]
Breast	CD44+/CD24-, ALDH1	Carrasco et al. [134]
Brain	CD133, CD44	Jackson et al. [135]
Lung	CD133	Lundin et al. [136]
Melanoma	CD20, CD133, CD271	Lang et al. [137]
Prostate	CD44+/CD24-, CD133, ALDH1	Sharpe et al. [138]

associated with poorer clinical outcomes [58, 62]. Another proposed gastrointestinal stem cell marker, doublecortin-like kinase (Dclk1), is highly expressed on cells in the stem cell zone of mouse gastric glands [63]. K-ras-induced chronic inflammation in K19-K-ras-V12 transgenic mice led to expansion of the Dclk1+ cell population during the development of high-grade dysplasia [64].

An alternative hypothesis suggests that gastric CSCs arise from bone marrow-derived mesenchymal stem cells—pluripotent adult stem cells that are recruited to peripheral organs in response to chronic inflammation. Their function is to assist in regeneration after failure of local stem cells [65]. Bone marrow-derived cells (BMDC) have been shown to repopulate gastric mucosa in response to chronic inflammation due to *H. Pylori* infection, and may contribute to carcinogenesis [66, 67]. In a mouse model infected with *H. pylori*, almost a quarter of high-grade dysplastic gastric lesions included BMDC [68]. These cells are thought to differentiate in the gastric mucosa by cell–cell fusion with local gastric epithelial cells and in the context of further chronic inflammation, induce EMT and the emergence of CSCs [65, 69]. It is important to note, however, that despite these provocative findings, the majority of dysplastic lesions do not arise from BMDC. Further studies are needed to fully explore the pathogenesis of gastric CSCs.

Gastric CSC markers

CD44

CD44 is a transmembrane glycoprotein expressed on leukocytes, endothelial cells, hepatocytes, and mesenchymal cells, and has a variety of physiological roles, including matrix adhesion, cell migration, and differentiation [70]. CD44 has been proposed as a mediator of signal transduction of oncogenic pathways such as the human epidermal growth factor receptor (HER) [71]. Cancer cells with high CD44 expression have enhanced resistance to reactive oxygen species as a result of increased glutathione synthesis and upregulation of antioxidant genes [72]. The first evidence of gastric CSCs was demonstrated with the self-renewal and heterogeneous lineage of a CD44+ subpopulation [11]. There are now numerous studies supporting CD44 as a marker for CSC in gastric cancer (Table 2; details of studies available in Supplementary Table 1). Two studies, however, were unable to demonstrate stem cell properties in the CD44+ purified subset of patient-derived gastric cancer cells, perhaps due to inappropriate microenvironments in mouse models [73, 74]. CSC populations have also been identified using a combination of CD44+ and other markers, including EpCAM [75, 76], CD54+ [77], and CD24 [78].

Notwithstanding the above-referenced works, CD44 is not a specific or sensitive marker for gastric CSC. The true CSC population has been estimated at <5 % of CD44+ cells [11], and CD44 is widely expressed on non-malignant tissue. Other markers are required in addition to CD44 to improve the specificity of CSC identification.

CD44 variants

CD44 is encoded by the 20-exon CD44 gene, with exons 1–5 encoding the constant region of the extracellular domain and exons 16–20 spliced together to form the standard isoform [76]. The variant exons 6–15 are subject to alternative splicing, and can be assembled in different combinations with the standard exon to generate variant isoforms [76]. CD44 variants (designated as CD44v) have been proposed as a more specific CSC marker than CD44 given their more restricted distribution pattern. Generally, expression of CD44v on gastric cancer cells correlates well with CD44 expression [32, 76].

There is only limited evidence of CD44v as a CSC marker. Lau et al. [76] showed that CD44v8-10 was the predominant CD44v expressed on CD44+ gastric cancer cells (79 % of CD44+ cells), and demonstrated self-renewal and heterogeneous lineage in serial transplants of CD44v8-10 in mouse models. Although the CD44v8-10+ fraction was more tumorigenic in mouse models, both the CD44v8-10+ and CD44v8-10- cells were able to form tumour spheres [76]. While CD44v appears to be a more specific marker for gastric cancer than CD44, more research is needed to elucidate its biological role and confirm CSC characteristics.

CD133

CD133 is a transmembrane glycoprotein plasma membrane protein found on embryonic epithelial structures and hematopoietic stem cells [79]. It has been proposed to function as an organiser of plasma membrane topology, and to play a role in maintaining appropriate lipid composition within the plasma membrane [80]. CD133 has been identified as a CSC marker in a variety of solid tumours (Table 1).

Most studies have identified CSCs using AC133, an antibody that detects a glycosylated epitope of CD133 [81]. Some authors have recommended caution in using CD133 as a marker to identify CSC, after they showed downregulation of CD133 epitopes (including the target of AC133) during differentiation but constant CD133 protein expression, suggesting that differentiated cells may express CD133, but with masked epitopes due to differential glycosylation [82]. Post-translational modification of CD133 may play a role in invasion and metastases, and may

Table 2 Summary of cancer stem cell markers in gastric cancer (in vitro and mouse model studies)

Stem cell characteristic	Marker	References
Self-renewal (serial transplantation in mouse models, or maintained in spheroid culture for weeks)	CD44+	Takaishi et al. [11]
	CD44v8-10	Lau et al. [76]
	CD133+	No studies identified
	ALDH1	Katsuno et al. [86], Nishikawa et al. [85]
Multi-lineage differentiation	CD44+	Takaishi et al. [11]
	CD44v8-10	Lau et al. [76]
	CD133+	Fukamachi et al. [139]
	ALDH1	Katsuno et al. [86]
Increased resistance to chemotherapy/radiotherapy	CD44+	Takaishi et al. [11], Zhang [78], Yoon et al. [104]
	CD133+	Zhu et al. [140]
	ALDH1	Zhi et al. [141], Nishikawa et al. [85]
Increased tumorigenicity (faster-growing tumours or smaller tumour seeding volume)	CD44+	Takaishi et al. [11], Song et al. [78]
	CD133+	No studies identified
	ALDH1	Zhi et al. [141], Katsuno et al. [86], Nishikawa et al. [85]
Attenuation of stem cell characteristics with knock-down model	CD44+	Takaishi et al. [11]
	CD133+	Zhu et al. [140]
	ALDH1	No studies identified
Up-regulated stem cell or mesenchymal markers	CD44+	Yu et al. [142]
	CD133+	Song et al. [127]
	ALDH1	Zhi et al. [141], Nishikawa et al. [85]

influence antibody binding by altering the epitope's accessibility [81].

Consequently, there is debate regarding the utility of CD133 as a CSC marker in gastric cancer. Although some studies have demonstrated CSC properties with the CD133+ subpopulation, several groups have found contrasting results, with CD133- cells able to initiate tumours [11, 73, 76] (Table 2; Supplementary Table 1).

ALDH1

Aldehyde dehydrogenase (ALDH) is a family of enzymes that play a role in cellular detoxification, differentiation, and drug resistance via oxidation of cellular aldehydes [83]. ALDH1 functions as a modulator of cell proliferation and stem cell differentiation, and is a marker of CSCs in a variety of cancers (Table 1). High ALDH1 activity confers resistance to chemotherapeutic agents [84]. ALDH^{high} cell populations are identified with the ALDEFLUOR assay or by the ALDH1 antibody, and have been shown to correlate with CD44 expression [85].

Katsuno et al. [86] demonstrated CSC properties, including self-renewal, heterogeneous lineage, and increased tumorigenicity, in ALDH1+ cells isolated from gastric cancer cell lines. Interestingly, they found that TGF- β inhibited the function of the CSC population, in

contrast to other cancers [86]. Studies demonstrating the CSC properties of ALDH1 in gastric cancer are summarised in Table 2 and Supplementary Table 1.

Other potential CSC markers

Numerous other molecules have been identified as potential gastric CSC markers, and these are addressed below. However, it is important to bear in mind that the evidence for these molecules as markers is either limited or contradictory. Further studies are thus required to either confirm or refute their utility as markers of the CSC population.

CD24 is a cell surface adhesion molecule expressed on leukocytes, normal gastric parietal cells, and intestinal stem cells [87]. CD24 expression is associated with aggressive clinicopathological features in gastric cancer, and facilitates cell migration and invasion in gastric cancer cells [87, 88]. Evidence for CD24 as a CSC marker in gastric cancer is conflicting. Zang et al. [78] found that the CD44+/CD24+ cell population isolated CSCs in gastric cancer cell lines, while Takaishi et al. [11] were unable to find evidence of CSC characteristics in a CD24+ population in spheroid and mouse models.

The epithelial cellular adhesion molecule (EpcAM) is a transmembrane glycoprotein detected in the majority of epithelial tissues, with roles in cell adhesion, signalling,

migration, proliferation, and differentiation [89]. EpCAM is over-expressed in gastric cancer and gastric cancer cell lines [85, 90]. Several studies have shown that gastric CSCs lie within the EpCAM+ population, with the EpCAM– population unable to form tumours in mouse models or tumour spheres [75, 76]. However, other, more specific markers are needed in addition to EpCAM, as the majority of gastric cancer cells are EpCAM-positive.

CD49f, a subunit of laminin receptors, has been used to isolate CSCs in prostate, breast, brain, and colon cancer [74]. Fukamachi et al. [74] demonstrated CSC properties of self-renewal, heterogeneous lineage, and chemotherapy resistance in the CD49f-selected cells from primary gastric cancer mouse xenografts. Further studies are needed to confirm CD49f stem cell properties.

CD54 [also known as intercellular adhesion molecule 1 (ICAM-1)] is an adhesion molecule essential for the arrest and transmigration of leukocytes out of blood vessels, and is widely expressed on immune, stromal, and malignant cells [91]. Decreased CD54 expression on resected gastric cancer is associated with poorer prognosis and increased risk of lymphatic spread [92]. CD44+/54+ cells isolated from primary gastric cancers and peripheral blood samples demonstrated superior tumorigenicity, multiple lineage capability, and self-renewal compared to CD44– or CD54– cells, suggestive of a CSC population in both the primary tumour and the circulation [77].

CD90 is expressed in bone marrow-derived mesenchymal stem cells, hematopoietic stem cells, and keratinocytic stem cells, and has been used to identify CSC populations in liver, breast, and brain cancer [93]. CD90+ cells isolated from patient-derived gastric cancer xenografts demonstrated self-renewal and a heterogeneous lineage [93]. CSCs obtained by preconditioning a gastric cancer cell line with chemotherapy displayed increased expression of CD90 as well as bonafide CSC markers [41].

CD71 (also known as the transferrin receptor) is a membrane protein highly expressed on myocytes, keratinocytes, hepatocytes, pancreatic cells, and erythroid precursors, with a physiological role in mediating the uptake of transferrin-iron complexes. CD71 has been proposed as a negative selection marker, with the CD71-negative subpopulation of a gastric cancer cell line displaying chemoresistance, self-renewal, heterogeneous lineage, and increased tumorigenicity in mouse models [94].

Finally, several transcription factors, including Sox2, Oct4, and Nanog are expressed on gastric stem cells and have been proposed as potential CSC markers. Gastric CSCs enriched by the side population assay or spheroid formation have higher expression of Sox2, Oct4, and Nanog than parental cells [12]. As discussed above, however, studies evaluating the association between clinicopathological variables with immunohistochemical

expression of Sox2 in resected gastric cancer have shown conflicting results [58, 59, 62, 95]. Similarly, although some studies have shown poorer prognosis associated with Oct4 expression in resected gastric cancer [96, 97], another large patient series found the opposite result [60].

Clinical implications of gastric cancer stem cells

Gastric CSC marker expression and patient prognosis

CSC marker expression in cancer tissue is emerging as a clinically relevant prognostic biomarker in the management of gastric cancer. Most studies have shown a correlation between advanced pathological features, such as tumour size, invasion, and metastatic spread, and expression of CSC markers. In addition, CD44 and CD133 expression was found to be an independent predictor of lower rates of disease-free survival (in resected gastric cancer) and overall survival (see Table 3). These findings support the preclinical evidence of CD44 and CD133 as CSC markers, as patients with tumours expressing these markers would be expected to have a poorer prognosis due to the CSC traits of chemo-radioresistance, increased tumorigenicity, and metastatic potential.

CD44 is expressed on up to 80 % of primary gastric cancer resection specimens [98] and is associated with more advanced clinicopathological features and poorer prognosis (Table 3). A meta-analysis of 18 studies examining CD44 expression in gastric cancer—albeit limited by significant methodological flaws, including a lack of qualitative analysis of included studies and considerable heterogeneity in pooled results—found statistically significant associations with advanced tumour stage [pooled odds ratio (OR) = 2.05, 95 % confidence interval (CI) 1.12–3.75, $P = 0.02$], tumor size (pooled OR = 1.42, 95 % CI 1.08–1.87, $P = 0.01$), and lymph node (LN) metastasis (pooled OR = 1.50, 95 % CI 1.14–1.98, $P = 0.004$) [99]. Although four studies have shown CD44+ expression to be an independent predictor of survival, it is important to note that the two largest case series did not show an impact of CD44 expression on overall survival [100, 101]. The heterogeneity in results is likely due to variation in experimental procedures and patient populations. A full table summarising all studies evaluating CD44 expression in gastric cancer is included in the supplementary materials (Supplementary Table 2).

Despite the contradictory preclinical data, the role of CD133 as a CSC marker is supported by numerous immunohistochemical studies in resected primary gastric cancer, showing a consistent association with numerous high-risk clinicopathological features and an independent

Table 3 Statistically significant clinicopathological and prognostic associations with CD44 and CD133 immunohistochemical expression in gastric cancer

Association	CD44 References	CD133 References
Intestinal histology	Mayer [32]	Wakamatsu [83]
	Hong [143]	Lee [146]
	Ghaffarzadehgan [144]	Nosrati [145]
Higher TNM stage	Nosrati [145]	Yu [147]
	Wakamatsu [83]	Zhao [148]
	Chen [33]	Hashimoto [149]
Larger tumour size/deeper invasion		Chen [33]
	Nosrati [145]	Yu [147]
		Zhao [148]
Lymphovascular invasion		Lee [146]
	Nosrati [145]	Zhao [148]
	Wang [98]	Chen [33]
Higher grade/poorer differentiation	Chen [33]	Jiang [150]
		Hashimoto [149]
		Chen [33]
Presence of distant metastasis	Mayer [32]	Chen [33]
	Chen [33]	Hashimoto [149]
Positive lymph nodes	Wakamatsu [83]	Yu [147]
		Zhao [148]
		Wakamatsu [83]
Poorer disease-free survival (multivariate)		Hashimoto [149]
	Mayer [32]	Lee [146]
		Hashimoto [149]
Poorer overall survival (multivariate)	Mayer [32]	Yu [147]
	Ghaffarzadehgan [144]	Zhao [148]
	Wakamatsu [83]	Wang [98]
	Chen [33]	Lee [146]
		Chen [33]
		Hashimoto [149]
	Wakamatsu [83]	

correlation with poorer disease-free and overall survival (Table 3; Supplementary Table 2). A recent meta-analysis found strong evidence that CD133 expression in resected gastric cancer was associated with poorer 5-year overall survival (OR = 0.2, 95 % CI 0.14–0.29, $p < 0.00001$), although it should be noted that all included studies were conducted in Asian populations, limiting the applicability to Western populations [102]. Furthermore, another recent study, which detected circulating CSCs using CD133 and ABCG2 as markers, found that the presence of peripheral blood CD133+ cells correlated with poorer prognosis, and that passage of isolated CD133+/ABCG2+ cells was possible in mouse models, showing self-renewal, heterogeneous lineage, and increased tumorigenicity [103].

CD44 variant expression appears to be more specific for malignant tissue. Expression of CD44 variants v5, v6, and v9, in resected gastric cancer is associated with adverse clinical outcomes, including worse overall survival, more

advanced tumours, and lymphovascular invasion (Table 4). A meta-analysis found that CD44v6 expression was related to LN metastasis (pooled OR = 2.26, 95 % CI 1.40–3.64, $P = 0.0008$), lymphatic invasion (pooled OR = 1.45, 95 % CI 1.05–2.01, $P = 0.02$), and venous invasion (pooled OR = 1.62, 95 % CI 1.20–2.18, $P = 0.001$), but not tumour stage (pooled OR = 0.68, 95 % CI 0.36–1.28, $P = 0.23$) [99].

ALDH1 expression has been shown to be associated with poor prognostic clinicopathological features in resected primary gastric cancer, although it is not significantly associated with poorer survival [83].

Targeting CSCs in gastric cancer

The CSC model has important clinical implications for cancer treatment. There is strong evidence that CSCs are resistant to traditional chemotherapy and radiotherapy, and

Table 4 Statistically significant clinicopathological and prognostic associations of CD44 variants in resected early gastric cancer

CD44 Variant	No. of cases	Statistically significant clinicopathological associations	Prognostic outcomes	Study
CD44v5	418	Diffuse classification Signet ring histology Higher differentiation Depth of invasion Lymphovascular invasion	Worse OS with CD44v5 expression*	Muller et al. [151]
CD44v6	103	Depth of invasion Positive lymph node metastases Presence of distant metastasis	No significant difference in OS	Hong et al. [143] (abstract only)
CD44v6	418	No significant associations	No significant difference in OS	Muller et al. [151]
CD44v6	62	Poorly differentiated Positive lymph node metastases Presence of distant metastasis	NS	Liu et al. [100]
CD44v6	201	Presence of vascular invasion Presence of distant metastasis	No significant difference in OS	Yamaguchi et al. [152]
CD44v6	135	Positive lymph node metastases Advanced TNM stage	NS	Okayama et al. [153]
CD44v6	155	Advanced TNM stage Presence of distant metastasis	No significant difference in OS	Xin et al. [154]
CD44v6	43	Depth of invasion Positive lymph node metastases advanced TNM stage	NS	Chen et al. [155]
CD44v6	98	Positive lymph node metastases	NS	Kurozumi et al. [156]
CD44v9	478	Higher TNM stage Positive lymph node metastases	NS	Yasui et al. [157]
CD44v9	24	Higher differentiation Intestinal classification	Worse OS with CD44v9 expression	Go et al. [158]
CD44v9	65	No significant associations	Higher risk of recurrence	Hirata et al. [159]

NS not studied, OS overall survival

* Statistically significant only in univariate analysis (not significant in multivariate)

are enriched in the residual tumour after such treatment [11, 78, 104, 105]. The CSCs subsequently renew the tumour bulk by developing treatment-resistant clones. Consequently, a specific and efficacious CSC-targeted therapy is needed in order to completely eliminate the cancer. These targeted treatments should be administered in conjunction with conventional chemotherapy/radiotherapy as a means to reduce the tumour bulk and minimise the risk of differentiated cancer cells acquiring CSC-like properties [2].

Targeting cancer stem cell surface markers

One proposed method of CSC-specific treatment is the use of drugs targeted at CSC surface markers. As discussed above, there are significant challenges with this approach given the widespread expression of these markers on non-malignant tissue and the rarity of CSCs in the tumour. Although there is promising data emerging from the

preclinical setting in the targeting of CD44, CD133, EpCAM, and CD90 (discussed below), the largest hurdle will be demonstrating safety and efficacy in vivo.

Methodologies targeting CD44 include anti-CD44 monoclonal antibodies [106] and anti-CD44 antibody or aptamer-labelled liposomes [107, 108]. The CD44 ligand, hyaluronic acid, has also been used to label nanocarriers and conjugates, with demonstrated efficacy in reducing CD44+ cells in pancreatic [109] and gastric cancer [110, 111]. Although these studies have shown promise in reducing CSC populations, the clinical utility of these agents may be limited by off-target toxicities [112, 113].

Similarly, CD133 has been successfully targeted in preclinical models by anti-CD133 antibody-cytotoxic conjugates in breast [114], ovarian [115], hepatocellular, and gastric cancer [116]. Smith et al. [116] developed a CD133-cytotoxic conjugate that inhibited the growth of gastric cancer cell lines in vitro.

The anti-EpCAM antibody MT201 has shown tumour suppression properties in preclinical studies in prostate and colon cancer, and has advanced to human trials [116, 117]. A phase I study demonstrated reasonable tolerability in combination with chemotherapy in heavily pretreated breast cancer, with further studies evaluating efficacy underway [118].

It may also be possible to reduce the CSC population through indirect targeting. Jiang et al. [93] noted that candidate CSC marker CD90 correlated with HER2 expression in gastric cancer. While chemotherapy enriched the CD90+ population in a primary cancer culture, a combination of chemotherapy and anti-HER2 treatment (trastuzumab) significantly reduced the CD90+ population and prevented tumour growth [93]. The underlying mechanism driving this result remains unclear, but is an interesting hypothesis to explain why some breast cancer patients with normal HER2 gene expression benefit from trastuzumab [119].

Targeting the cancer stem cell signalling pathways

CSCs are formed as a result of aberrations in important normal stem cell signalling pathways, such as Hedgehog (HH), Notch, and Wnt [13], which have been shown to be important potential targets for treating CSCs [120].

The Wnt/ β -catenin pathway plays a physiological role in balancing the proliferation, differentiation, and “stemness” of cells, with over-activation leading to tumorigenesis [121]. It is an important pathway in the regulation of CSCs, and many of the cell surface markers discussed above, including CD44, CD24, and EpCAM, are Wnt targets [122]. Blockage of the Wnt pathways reduces the self-renewal capacity of gastric cancer tumour spheres [123]. One study demonstrated that gastric cancer cells over-expressing Wnt-1 resulted in larger mouse xenograft tumours compared to those with controls cells, with increased expression of CSC markers such as CD44 [124]. When salinomycin was used to suppress Wnt and β -catenin expression, the tumours were smaller, with reduced CSC populations [124]. Another group showed that the disruption of Wnt signalling in CD44+ selected gastric cancer cells using a Wnt-1 antagonist (Dickkopf-1) delivered by adenovirus serotype 5 served to inhibit cancer cell survival, colony formation, and invasion [125]. These agents are awaiting clinical validation.

Aberrant activation of the HH pathway causes neoplastic transformation in a variety of tumours, including gastric cancer [126]. HH signalling maintains the CSC phenotype, and in vitro targeting of the HH pathway reduces the tumorigenicity and invasive capability of gastric cancer spheroids [104] and reverses chemoresistance [127]. Yoon et al. [104] retrospectively performed immunohistochemistry on gastric cancer samples from a negative randomised phase II trial examining the addition of an HH inhibitor

(vismodegib) to chemotherapy in gastric cancer, and found improved survival in patients expressing CSC markers (CD44). This exciting finding is the first evidence of CSC expression as a predictive biomarker in gastric cancer, and demonstrates the immediate clinical applicability of targeting CSC pathways as an adjunct to chemotherapy.

Notch signalling has an important role in gastric epithelial stem cell homeostasis, and has been implicated in gastric cancer tumorigenesis [128–130]. Gamma-secretase inhibitors that block the Notch pathway act to reduce CSC markers and cancer growth [131]. They are currently in early clinical trials, but may be limited by toxicity due to their non-specific activity.

Conclusions

Gastric cancer continues to be a highly lethal malignancy, despite the use of multimodal treatment approaches. Strong preclinical and clinical evidence supports the existence of gastric CSCs. CSCs are a small population of tumour cells that, enriched by chemotherapy, provide a source for the reconstitution and spread of the tumour. The failure of conventional treatments to achieve a significant increase in survival rates despite improvements in tumour response can thus be explained by the CSC model.

Gastric CSCs have important clinical ramifications. Expression of CSC markers has been shown to be a clinically relevant biomarker in resected gastric cancer. The cancer stem cell model also provides a new paradigm for managing patients with gastric cancer. Preclinical studies have shown the value of targeting validated and experimental CSC pathways and markers to reduce their stem cell-like characteristics, thus enabling a more complete treatment of gastric cancer. We await the results of further studies to evaluate the efficacy and safety of incorporating CSC treatments into clinical care.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding Funding sources include the Cancer Institute New South Wales for the CONCERT-Translational Cancer Research Centre and Illawarra Health and Medical Research Institute, University of Wollongong.

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