The identifications and clinical implications of cancer stem cells in colorectal cancer

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Abstract

Cancer stem cells (CSCs) are cancer cells that are responsible for initiation, progression, metastasis and recurrence in cancer. The aim of this review is to analyse the markers for identifying of CSCs in colorectal carcinoma as well as the prognostic and therapeutic implications of these markers in the cancer. CSCs are insensitive to the current drugs regimens. In colorectal carcinoma, markers including Nanog, Oct-4, SOX-2, Lgr-5, CD133, CD24, CD29, ALDH1, EpCAM, CD44, CD166 and CD26 are commonly used for the identification and isolation of CSCs. In addition, ALDH1, CD24, CD44, CD133, CD166, EpCAM, Lgr-5, Nanog and SOX-2 could have clinical roles in predicting pathological stages, cancer recurrence, therapy resistance and patients' survival in patients with colorectal carcinoma. In light of the current knowledge of CSCs in colorectal carcinoma, novel potential therapeutic strategies such as development of monoclonal antibodies or immunotoxins and targeting various cell surface molecules in colorectal CSCs and/or components of signalling pathways have been developed. This could open new opportunities for the better management of patients with colorectal carcinoma.

Keywords: Cancer stem cells; colorectal cancer; carcinoma; markers; therapy

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in female, with an estimated 1.4 million incidences and 693,900 deaths occurred in the year 2012 throughout the world [1]. Failure of treatment of patients with CRC could be attributed to the escaped residual microscopic carcinoma after surgery, which later initiates the metastatic process [2]. In principle, these residual cancer cells are eliminated by post-operative chemotherapy and/or radiotherapy. However, the presence of therapy resistant cancer cells population limits the success of these treatments [3, 4]. Genetic, epigenetic and functional heterogeneity of cancer cells support the existence of these therapy resistant cancer cells in patients with CRC [5-7]. These small fractions of cells within the cancers are called cancer stem cells (CSC). These cancer stem cells are capable of initiating, maintaining and development of the cancer growth [3, 8]. Also, CSCs have self-renewal capacity and responsible for developing functionally and morphologically diverse cells including therapy resistance and metastatic cell populations [3].

CSCs have been implicated in colorectal carcinogenesis for a long time though their existence has only been recently demonstrated experimentally [9, 10]. In view of the importance of CSCs in CRC, we aimed to review the markers for identifying of CSCs in CRC as well as the prognostic and therapeutic implications of these markers in CRC.

Identification of cancer stem cells

By definition, CSCs are the cells, which have the capacity to drive carcinogenesis through long-term production and self-renewal of differentiated, non-tumorigenic progenies [11]. It was also reported that chemo-radiotherapy resistant CSCs has greater potential of tumour initiation and stimulated the regrowth of cancer after a therapeutic treatment [12-15]. The existence and the identity of CSCs have been reported first time in hematopoietic cancers

[16]. Thereafter, CSCs from many solid cancers such as arising from breast, brain, prostate, head and neck etc. were also identified [12, 13, 17].

The current gold standard for defining CSC "stemness" is to show their ability to transfer disease into immuno-deficient mice at a limited dilution [14, 15]. This type of xenograft assay involves fluorescence-activated cell sorting (FACS) of single cancer cell that has the putative CSC properties and demonstrating its ability to develop a new cancer similar to the original cancer [14, 15, 18]. The limitation of this method is partly related to the difficulties to discriminate between CSC and non-CSC populations of cancer cells. Also, the difference between the microenvironment of the original cancer and the transplanted recipient may have impact on the function of CSCs [19]. Thus the identification and isolation of cancer stem cell is still a matter of debate due to lack of unique methods for isolation and identification as well as their complex biology [9, 10].

Identification of CSCs in CRC (Table 1)

Genes such as *Nanog, Oct-4 and SOX-2* are responsible for the pluripotency of cells and are commonly considered to be the surrogate markers for cancer stem cells [20, 21]. Nanog, a homeobox protein encoded by *Nanog*, is a transcription factor and regulates the stem cell properties especially self-renewal pluripotency of cell [22]. Matsuoka and colleagues showed that nanog was positive in 28 (10%) of 290 gastric cancer tissues [23]. In colorectal cancer, Meng and colleagues has highlighted the importance of Nanog in the maintenance of cell proliferation, invasion and motility of CRC cells as well as its contribution to the epithelial mesenchymal transition (EMA) in the development of colorectal cancer [24].

Oct 4 (a member of POU family) contributes to the self-renewal ability and inhibits the genes responsible for differentiation as well as to enable the self-renewal ability of stem

cells [25, 26]. Padin-Iruegas and colleagues demonstrated that Oct4 mRNA was present in the peripheral blood of patients with metastatic colorectal cancer [27].

Sex determining region Y (SRY)-box 2 (SOX-2) is a stem cell marker and plays crucial roles in the maintenance of cell pluripotency and self-renewal [28-30]. In addition, it has been reported that SOX-2 plays an important role in the maintenance of self-renewal of CSCs [31]. Knockdown of *SOX-2* and *Oct4* reduced the tumour size in oral cancer in immunodeficient mice [32]. Furthermore, SOX-2 was found positive in 159 (55%) of 290 gastric cancers [23]. In colorectal cancer, SOX-2 has been used to identify the CSCs in many studies [33-35].

O'Brien and his group noted that CD133 positive human cancer cells were able to produce cancer of similar morphology to the original one in immuno-deficient mice whereas the CD133 negative cells were unable to initiate cancer growth [15]. CD133 has been used to study 501 CRC on tissue microarrays in colorectal cancer [34].

Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr-5) positive cells (Lgr-5+) have the characteristic features of CSCs in CRC [36, 37, 38, 39, 40]. Schepers and colleagues demonstrated that some cells within the mouse colonic adenoma (5-10%) were Lgr-5+ cells. These cells were responsible for self-renewal and production of differentiated Lgr-5− colonic adenoma cells [41]. It was reported that patients with colorectal cancer expressing high Lgr-5 had 10-fold higher risk for cancer relapse than patients with low expression of Lgr-5 [39]. In addition, it has been demonstrated that Lgr-5+ cells derived from patients with colorectal carcinoma have the potential of CSC as they showed high number of spheroid formation in culture conditions [42]. Therefore, Lgr-5 has the potential to be used as a surrogate marker for the identification of CSCs in colorectal cancer.

Cluster of differentiation 24 (CD 24), also called heat stable antigen 24 (HAS) or signal transducer 24, is a glycoprotein and expressed at the cell surface of lymphocytes [43].

Rowehl and colleagues reported the establishments of CRC's cancer stem cells using in vitro and in vivo mouse model from liver metastasis of patients with colon cancer [44]. This study also demonstrated that CD24+ cells were highly tumorigenic and clonogenic with increased stemness, pluripotency and exhibited resistance to therapy [44]. Sahlberg and colleagues reported that colon cancer cells expressing CD24, CD133 and CD44 act as CSCs and was associated with radiation resistance in colon cancer cells [45]. Thus, CD24 can be used as a putative marker for CSC isolation and identification in CRCs.

CD29, also called integrin beta-1 protein, is encoded by *ITGB1* gene. It plays a key role in cell adhesion and various cellular processes like embryogenesis, haemostasis, tissue repair, immune response and cancer metastases [46]. CD29 is reported to be a surface marker for the highly proliferative site of human colonic crypt and thereby CD29 positive cells could be used as a marker for stem cell type in human colon [47]. In addition, high expressions of CD29 were noted in human colon CSCs and these cells acted as tumour initiator/cancer stem cells in mouse colonic carcinoma [48]. Another study has identified that colon CSCs with phenotypic fractions of CD^{29+}/CD^{133+}cells exhibited distinct proliferation, differentiation and self-renewal properties [49]. These studies suggest that CD29 can be used as a surface marker in identifying CSCs in colon cancers.

Aldehyde dehydrogenase isoform 1 (ALDH1) is an isoform of aldehyde dehydrogenase enzyme and catalyses the conversion of aldehyde to carboxylic acid [50]. This enzyme is commonly used as a surrogate marker for the identification of non-cancer stem cells as well as CSCs in different cancers including breast cancer, pancreatic cancer, prostatic cancer, lung cancer, leukaemia, multiple myeloma, melanoma and liver cancer [11, 17]. Studies have noted that ALDH1 is a potential CSCs marker in CRC [51, 52]. Increased ALDH1 expressions in colon cancer tissue samples were associated with poor differentiation (high grade) and presence of metastasis [53, 54].

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein, mediates homotypic cell-cell adhesion in the epithelia and regulates cell proliferation, differentiation, migration and cell-signalling [55, 56]. This cell surface marker has the potential to be used as a diagnostic marker for detecting carcinomas [55]. Roy and co-workers have isolated colonic CSCs using EpCAM, CD133 and CD44 cell surface markers in the xenograft cancer stem cell mice model [57].

CD44 is a cell surface glycoprotein encoded by *CD44* gene and regulates cell-cell interactions, cell adhesion and migration [58]. CSCs from different cancers including colon cancer, breast cancer, pancreatic cancer, head and neck cancer, hepatocellular carcinoma, and non-small cell lung cancers have been identified and isolated using CD44 [59]. It was reported that CD44+ colorectal cancer cells exhibited higher *in vitro* clonogenic properties and showed higher *in vivo* tumorigenicity when compared to that of CD44- cells [60]. Furthermore, CD44+ CRC cells displayed the phenotypic and morphological characteristics of cancer following serial transplantation into immunodeficient animals [60]. These CD44+ cells maintained their stemness by activation of tyrosin kinase receptor c-Met in colon cancer [60]. Dalerba and colleagues demonstrated that triple positive surface phenotype ($EpCAM^{high}$/CD44+/CD166+) could be used for the as a precise method for identification of colonic CSCs [59].

Wang and colleagues noted that CD44+ colon cancer cells displayed more aggressive proliferation, higher colony formation, less sensitive to apoptosis signals and more resistance to therapy when compared to that of CD44- cells [61]. However, studies noted controversial role of CD44 in the pathogenesis of colorectal cancer [62, 63]. Dallas and co-workers noted that down regulation of CD44 increased migration and metastasis of colon cancer cells [62]. Also, loss of CD44 was noted to be correlated with aggressiveness of colon carcinomas was

reported by Ylagan and colleagues [63]. Thus, further studies are needed to confirm the role of CD44 in the maintaining of stemness of colon CSCs.

CD166, also called activated leukocyte adhesion molecule (ALCAM), is a transmembrane glycoprotein in the immunoglobulin superfamily. It is encoded by *ALCAM* and characterized by the five extracellular immunoglobulin-like domains [64]. CD166 is expressed high in colon cancer, lung cancer, breast cancer, melanoma and prostate cancer cells [65]. Several studies demonstrated the identification of colorectal CSCs cells using CD166 as a cell surface marker [66, 67]. For instance, Mărgaritescu and colleagues reported the identification of colon carcinoma stem cells using CD133/CD166/Ki-67 triple positive phenotype in immunofluorescence techniques [66]. These results were in consensus with the earlier findings of Dalerba and co-workers in which colonic CSCs were isolated using CD166 [67].

CD26 is a cell surface glycoprotein which is expressed in a variety of cell types including endothelial cells, epithelial cells and T lymphocytes with various biological functions [68, 69]. In colorectal cancer, Pang and colleagues reported that $CD26^+$ cells have more adhesion tendency to fibronectin and type 1 collagen in compare to $CD26^-$ cells [70]. Furthermore, they found that transient knockdown of CD26 in $CD26^+$ cells decreased the migratory and invasive capacity of $CD26^+$ CSCs.

Prognostic value of cancer stem cell markers in colorectal cancer

CSCs can regulate cancer invasion, distant metastases, therapy resistance in CRC as well as contributed to the cancer recurrence of patients with CRC [71]. Taken together, the markers for CSCs could potentially have important implications in the prognosis of patients with colorectal carcinomas (Table 2).

ALDH1

High expression of DNA repair mechanism, aldehyde dehydrogenase isoform 1 (ALDH 1) and other molecular pumps such as ATP-binding cassette transporter (ABC-transporter) in CSCs contribute to overcome the effect of chemo-radiotherapy in colorectal cancer [67, 71]. Increased ALDH1 expressions in colon cancer tissue samples were associated with advanced clinical stage [53]. Lugli and co-workers demonstrated that overexpression of ALDH1 protein in primary colorectal adenocarcinoma tissues (n=1420) via immunostaining was associated with high pathological grade and poor survival of the patients [54]. Similarly, Fitzgerald and colleagues reported that high ALDH1 protein expression, detected by immunohistochemistry, in stage IV colorectal adenocarcinoma tissues (n=30) was correlated with poor survival of the patients [72]. Recently, Deng and co-workers reported that patients with rectal cancer (n=64) receiving preoperative radio-chemotherapy showed high expression levels of different CSCs markers including ALDH1 by immunostaining [73]. They noted that high ALDH1 expression in patients with post-neoadjuvant therapy correlated with cancer relapse, distant metastasis and poor prognosis in patients with rectal cancer [73]. Also, Goosssens-Beumer and co-workers studied the expression of ALDH1 in a large cohort of patients (n=309) with CRC by immunohistochemistry was significantly correlated with poor clinical outcome of the patients [74]. Furthermore, Kahlert and colleagues noted that ALDH1 nuclear expression was associated with shortened overall survival of patients with CRC [75]. Therefore, these studies indicated that ALDH1 acts as a strong prognostic marker in patients with CRC.

CD24

CD24 is a glycosylphosphatidylinositol anchored membrane protein and act as an adhesive molecule on the activated endothelial cells and platelets [76-79]. Studies

demonstrated that CD24 is a potential prognostic marker in various cancers such as ovarian cancer, non-small cell lung cancer, prostatic cancer, gastric adenocarcinoma and breast cancer [80-84].

Weichert and colleagues showed strong cytoplasmic CD24 expression in colorectal cancers and the expression of CD24 was associated with shortened patients' survival in patients with colorectal cancers [85]. Also, Choi and co-workers showed that CD24 expression was related to the histological grade and size of the colorectal cancer [86].

Seo and colleagues examined 174 stage II and Stage III CRC tissues by immunohistochemistry techniques and noted that positive expression of CD24 was correlated with the poor survival of patients with CRC [87]. On the other hand, Ahmed and colleagues examined whole tissues sections of colorectal adenoma (n=10) and CRC tissue microarray samples from 345 patients using immunohistochemistry did not find the prognostic implication of CD24 in patients with CRC [88]. In this study, positive immunoreactivity was noted in 90% (9/10) of colorectal adenoma and 91% (313/345) of CRC tissues samples. This lack of association with CD24 and patients' outcome in colorectal cancer might be attributed to the poor representation of cancer cells in the tissue microarray sections. Taken together, more research with large number of CRC tissues samples as well as functional studies are imperative to establish the prognostic value of CD24 in CRC.

CD44

Huh and colleagues demonstrated that CD44 was expressed in 100% (74/74) of CRC and its expression was significantly associated with depth of invasion and lymph node involvement [89]. Also, Wielenga and colleagues reported that CD44v6 overexpression in frozen tissue sections obtained from CRC patients could identify patients who are highly predispose to develop distant metastasis [90]. Furthermore, they demonstrated that CD44s

expression can be an independent prognostic factor for advanced CRC, especially in stage IV disease. In addition, Choi and colleagues reported that CD44 expression was significant correlated with tumour size in patients with colorectal adenocarcinoma (n=523) [86]. Furthermore, Ngan and co-workers demonstrated that loss of CD44 protein expression in CRC tissues sample (n=140) in immunostaining strongly correlated with poor survival and indicated that CD44 loss has worst impact on patients prognosis [91].

Despite all the positive correlations noted, Morrin and Delaney examined CD44v6 protein and mRNA expression by immunohistochemistry and reverse transcriptase polymerase chain reaction in 88 colorectal cancer tissues and found no correlation of CD44v6 protein and mRNA expression with cancer stages, grade, differentiation or survival of the patients [92]. These conflicting results might be associated with heterogeneity in cancer cells from different populations and varying samples sizes in the study population.

Furthermore, Jing et al noted that CD44 mRNA expression was higher in colorectal cancer metastases in liver when compared to the primary cancer in a cohort of 36 patients. Also, the expression was an independent prognostic factor [93].

CD133

CD133 is a known stem cell marker and is widely used as a marker for identifying colon CSCs [94-96]. Saigusa and co-workers investigated the expression level of CD133 gene and protein in patients with rectal cancer (n=33) after chemoradiation therapy. They noted that increased expression of CD133 both in gene and protein level is correlated with distant recurrence and poor prognosis [35]. Also, Kemper and colleagues noted that CD133 mRNA expression predicted poorer survival in 90 patients with stage 2 colorectal carcinomas [42]. CD133 mRNA was noted to higher in hepatic metastases from patients with colorectal carcinoma when compared to the primary cancer [93].

On protein expression level, Choi and colleagues studied CD133 protein expression in CRC tissues (n=523) by immunohistochemistry and found that there was significant relation of CD133 expression with advanced T stage cancer [86]. Also, Jao et al. investigated the protein expression of CD133 in colonic adenocarcinoma (n=157) and rectal adenocarcinoma (n=76) tissues samples by immunohistochemistry and noted that the cytoplasmic expression of CD133 protein was significantly associated with cancer local recurrence, survival and cancer regression after concurrent chemo-radiotherapy [97].

CD133 mRNA expression in liver tissues with metastatic colorectal cancer (n=50) as studied by quantitative real-time polymerase chain reaction showed that CD133 expression was significantly correlated with poorer survival of patients with CRC [98]. In addition, Horst and colleagues examined CRC tissues samples (n=57) by immunohistochemistry and demonstrated that high CD133 protein expression in an independent prognostic factor and correlated with poor survival time of patients with CRC [99]. Also, the group noted that high CD133 expression correlates with synchronous liver metastasis [100]. Furthermore, Kojima and co-workers studied CD133 protein expression by immunohistochemistry in CRC tissues (n=189) and reported that high CD133 expression was associated with shorter recurrence free survival and also with poor survival of patients with CRC [101].

In the literature, a number of studies examined the role of CD133 mRNA expression in peripheral blood samples obtained from patients with CRC to evaluate the prognostic value of CD133 in CRC patients. High CD133 mRNA expression in the peripheral blood of patients with CRC (n=100) was correlated with recurrence of CRC and can be used as independent prognostic factor in CRC [102]. In addition, Iinuma and colleagues studied the expression of carcinoembryonic antigen (CEA), cytokeratins (CK19, CK20) and CD133 in peripheral blood samples (n=735) obtained from different stages of CRC by real-time reverse transcription polymerase reaction assay [103]. They reported that overall disease free

survival of patients with CRC that are positive for CEA/CK/CD133 (especially in stage III cancers) was significantly poorer when compared with those who were negative for CEA/CK/CD133 [103]. Conversely, Gazzaniga and colleagues showed that the expression of CD133 mRNA in circulating tumour cells isolated from peripheral blood of patients with metastatic CRC (n=45) had no correlation with overall outcome of the patients [104].

Despite having a conflicting single study, majority of the studies supports the potential of colon CSCs marker CD133 as prognostic marker and more validation is required for its future use in clinical setting.

CD166

CD166 expression has been reported to be correlated with the pathogenesis of various cancers including melanoma, breast, prostatic, oesophageal, ovarian, urinary bladder, and colorectal cancers [105-111]. In colorectal cancer, Weichert and colleagues demonstrated that CD166 protein expression, as detected by immunohistochemistry, in colorectal cancer (n=111) was significantly associated with the survival time of patients with CRC [110]. They also noted that CD166 frequently upregulated in colorectal cancer and can be act as independent prognostic marker in progression of the cancer [110]. In addition, Horst and co-workers studied the expression of CSCs markers CD133, CD44 and CD166 in CRC (n=110) by immunohistochemistry and noted that these CSCs markers had significant prognostic implication in the prognosis of patients with CRC [100]. Furthermore, Sim and colleagues examined preoperative chemo-radiotherapy treated colorectal adenocarcinoma (n=112) by immunohistochemistry and noted that the expression of CD166 protein was correlated with cancer regression and poor patient prognosis [112]. These studies imply that CD166 is a key regulator in maintaining stem ness in colon cancer cells and it has the potential to be used as a prognostic maker for the clinical management of CRC patients.

EpCAM

Colon CSCs marker, EpCAM, has been reported to overexpress in many human cancers including colorectal cancer and has important role in cancer pathogenesis and prognosis [113-115]. Went and colleagues examined colon cancer tissues microarrays (n=1186) by immunohistochemistry and noted that high expression of EpCAM was significantly associated with higher grade colorectal cancer [114]. Zhou and co-workers studied the expression of EpCAM and Wnt/β-catenin in colon cancer (n=50) and non-neoplastic intestinal mucosae (n=20) by immunohistochemistry and noted higher expression of EpCAM in colon cancer [116]. They also reported high EpCAM expression was related to lower survival rate of patients with CRC [116]. On the other hand, Lugli et al demonstrated that reduced EpCAM expression was associated with tumour invasion, lymph node metastasis and high tumour grade [54]. Other studies demonstrated that loss/reduced expression of EpCAM was correlated with poor survival and cancer recurrence in patients suffering from CRC [74, 117, 118]. Therefore, more studies are needed to confirm the prognostic role of EpCAM in CRC.

Lgr-5

Lgr-5 overexpression has been reported to play an active role in regulating pathogenesis of colorectal cancer [119]. Takahashi and colleagues illustrated that high expression of Lgr-5 was related with lower disease free survival and presence of metastases to lymph node and liver [40]. Also, Liu and co-workers investigated Lgr-5 mRNA and protein expression in primary colon cancer tissues (n=366) and xenograft mice tissues (n=40) by real-time polymerase chain reaction and immunostaining respectively [121]. They found that Lgr-5 protein and mRNA significantly overexpressed in tissues from patients with CRC and correlated with higher cancer stages and poorer patients' survival [121].

Wu and colleagues reported that Lgr-5 protein expression in CRC (n=192) as detected by immunohistochemistry was significantly overexpressed when compared to that of non-neoplastic mucosae [120]. They also noted that higher expression of Lgr-5 protein was associated with higher histological grade, invasion, lymph node metastasis, distance metastasis and poorer survival of patients with CRC [120]. In addition, Hsu and colleagues demonstrated that high expression level of Lgr-5 was correlated with shorter disease free survival and shorter cancer-specific survival of patients with CRC [123]. They reported that patient with low expression of Lgr-5 showed better response than patients with higher expression of Lgr5 towards 5-FU-based treatment. Furthermore, Saigusa and colleagues demonstrated that Lgr-5 expression was highly expressed in specimens obtained from patients with poor pathological response and cancer recurrence [122]. They also found that patients with higher expression of Lgr-5 showed a significantly lower recurrence-free survival.

A meta-analysis carried out by Han and co-workers revealed that Lgr-5 overexpression was correlated with poor patients' survival suffering from CRC [124]. Overall, Lgr-5 is proposed to be an efficient prognostic marker for patients with colorectal cancer

Nanog

Xu and colleagues examined Nanog mRNA and protein expression in CRC (n=360) by real-time polymerase chain reaction assay and immunohistochemistry and the expressions were correlated with high histological grade, advance cancer stages as well as presence of lymph node and liver metastases in patients with CRC [125]. Also, Meng and colleagues found that higher expression of Nanog was associated with shorter survival or recurrence free survival [126]. Their meta-analysis also showed that Nanog is potential independent

prognostic factor of the outcomes of CRC patients. Nevertheless, Saiki and co-workers described that there was no correlation of Nanog mRNA expression with clinicopathological parameters of CRC (n=79) [33]. Thus more studies with large number of samples are needed to establish the prognostic role of Nanog in CRC.

SOX-2

Saigusa and colleagues examined the expression pattern of SOX-2 both at mRNA and protein level in 33 patients with rectal cancer after chemoradiation therapy by RT-PCR and Immunohistochemistry. They found that both the mRNA and protein for SOX-2 are overexpressed in all these patients. They also noted that higher expression of SOX-2 is correlated with poor disease-free survival and distant recurrence [35]. Also, Lundberg and colleagues noted that the expression of Sox-2 of 441 CRC by immunohistochemistry and noted that SOX-2 was expressed in 11% of the CRC and the expression was related to *BRAFV600E* mutation. SOX-2 expression was noted in the liver metastases of the patients with SOX-2 positive colorectal carcinomas [127].

Oct 4

Matsuoka and colleagues demonstrated that Oct3/4 was expressed in 129 (44%) of 290 gastric cancers and noted the correlations of the protein expression with prognosis of patients with gastric cancers [23]. In colorectal cancer, Saigusa and colleagues reported higher expression of Oct 4 in patients with rectal cancer after treatment with chemoradiation (n=33) was correlated with poor survival and distant recurrence [35].

Therapeutic implication of CSCs in CRC

Conventional cancer therapies can eradicate the cancer mass partly and could make the diseases more aggressive through recurrence and metastasis [128]. The principal limitation of current chemo-radiotherapy is that they only eliminate differentiated cancer cells but insensitive to the CSCs [129]. CSCs are the population of cancer cells which are responsible for the therapy resistance, cancer relapse and distant metastasis [129, 130]. These phenomena in turn confer more complications to the cancer patients in the course of disease. Thus, the development of treatment modalities targeted both conventional cancer cells and CSCs has greater translational implication in clinical setting for the better management of cancer.

The identification of putative CSC markers and the underlying signalling pathway they involved are critical for the development of novel therapeutic approaches. Also, the drug induced toxicity would be minimized by developing therapies targeting specific molecules or the pathways that are active in CSCs [131]. To achieve these goals, the prospective therapeutic strategies to specifically target CSCs which are under developments includes: (i) the eradication of CSCs by targeting selective marker expressed on the CRC's CSCs and (ii) the inhibition or interference of CSC-specific pathway (Fig.1).

Colon CSCs eradication targeting cell surface markers

Monoclonal antibodies/immunotoxins specific for the cell surface molecules of CSCs have the potential to eliminate the target CSC selectively [132, 133]. It was demonstrated that the therapeutic agents targeting cell surface markers e.g. CD133, CD44, CD26, CD29, EpCAM etc. could potentially eliminate CSCs, which in turn has the capacity to repress tumour size, reduce the metastatic potential of cancer cells and to decrease the cancer cell resistance to chemotherapy [134-138]. For example, CD133+ colon CSCs exhibited

resistance to the conventional chemotherapeutic agents (e.g. 5-fluorouracil and oxaliplatin) by increased secretion of cytokine IL-4 and escaped the apoptotic insults caused by the treatment [137, 139]. Importantly, colon cancer cells treated with 5-fluorouracil, oxaliplatin and monoclonal antibodies to IL-4 remarkably augmented the antitumor activity of the treatments [137, 139]. Dallas and colleagues reported that chemo-resistance fraction (CD133+ and CD44+) of HT29 CRC cells showed increased expression of Type 1 insulin-like growth factor receptor (IGF-IR) [135]. Treatment of these therapy resistant cells with IGR-IR monoclonal antibody caused significant inhibition of tumour growth in murine xenograft model [135]. In addition, treatment of patients with stage III CRC (n=189) with monoclonal antibody against EpCAM (colon CSCs marker) improves the cancer free survival and prolongs the cancer remission in patients with CRC [140].

Studies demonstrated that monoclonal antibodies specific for CD24 cell surface marker significantly inhibited the colon cancer growth and tumorigenic potential both *in vitro* and *in vivo* mouse model [136]. Also, down regulation of CD24 expression using short hairpin RNA (shRNA) retarded tumorigenicity in human cancer cell lines in culture and athymic mice [136].

Down regulation of CD29 by antisense oligonucleotide inhibited human colon cancer cell (HT29) migration *in vitro* and hepatic metastasis *in vivo* [134]. Park and colleagues reported that barberine (an alkaloid natural product) inhibited the migration of human colon cancer cells (HCT116 and SW-480) by reducing CD29 (integrin β 1) expression [134]. They noted that barberine treatment induce AMP-protein kinase signalling pathways in colon cancer cells, which in turn reduce the CD29 protein level and decreased the phosphorylation of CD29 targets [134]. In addition, Kanwar demonstrated that treatment of human colon cancer cells with diflourinated-curcumin in combination with conventional chemotherapy (5-flurouracil and oxaliplatin) significantly reduced the CD44 and CD166 population [141].

This treatment caused cancer growth inhibition, induction of apoptosis and disintegration of colonospheres [141]. Therefore, therapeutic strategies targeting cell surface markers of colon CSCs or their downstream signalling partners in combination with conventional therapy has the emerging potential to efficiently manage progression of CRC.

CSC elimination by targeting the signalling pathways

Activation of Notch, Wnt/β-catenin, TGF-β and Hedgehog signalling pathways have been reported to be contributed to the chemo-radiotherapy resistance of CSCs in cancer treatment [142, 143]. It was demonstrated that inhibition of these pathways by chemical intervention increased the sensitivity of CSCs to chemotherapy [131].

γ-secretase inhibitors have the potentials to inactivate Notch signalling and can be used to develop therapeutic strategies for the treatment of patients with CRC [144]. Constitutive activation of Wnt/β-catenin pathways in colon cancer makes this pathway as an important target for therapy development [144]. Deregulation of this pathway by inhibiting β-catenin accumulation and/or expression, and disrupting its interaction with other components has been reported to reduce colon cancer growth both in vitro and in vivo xenograft mouse model by Green and colleagues [145]. They treated colon cancer cell (SW-480) implanted mice with different concentrations of β-catenin antisense oligonucleotides and they noted dose-dependent tumour growth inhibition when compared to the scrambled control β-catenin oligonucleotides group [145]. van de Wetering M and co-workers reported that a small compound called inhibitor of Wnt production (IWP) has the potential to disrupt Wnt/β-catenin pathway by inhibiting porcupine (a membrane bound acetyl transferase) activity, which is essential for the production of Wnt protein [146].

Chen and colleagues illustrated that Sonic Hedgehog inhibitor (cerulenin, cyclopamine and itraconazole) significantly induced apoptosis, decreased cell proliferation,

inhibited spheres formation and reduced the expression of stemness factors in colon cancer HCT116 cells [147]. These inhibitors remarkably inhibited colitis-induced colorectal carcinogenesis by targeting cytokine IL-6 signalling in both culture and xenograft model of the cancer [147].

These studies indicates the effective repression of CSC activities in CRCs by targeting key signalling pathways and this has further implications in future targeted therapies in patients with CRC.

Concluding Remarks

Identification of cancer stem cells in colorectal carcinoma based on their surface markers could help in isolation as well as predicting of aggressive clinical behaviour, resistance to therapy, detection of cancer recurrence, survival and in the development of advanced cancer therapies. Newly identified CSC markers in colorectal cancer in combination with the existed markers could help in therapy selection and optimize the post treatment surveillance of patients.

Emerging therapeutic tools based on specific properties and functions of CSCs inside the bulk of a colorectal cancer could be useful for improved clinical outcomes. In future, potential improvement in management of patients with CRC could be achieved with the combination of CSCs targeted therapies with other anti-cancer therapies such as chemotherapy, radiation, molecular targeted therapy and immunotherapy, etc. Therefore, in-depth understanding of the biology, function, identification and clinical applications will help to achieve more effective management of patients with colorectal cancer.

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Figure Legends

Figure 1: Implication of cancer stem cells (CSCs) as a therapeutic target in colorectal cancer treatment. (A) Metastatic cancer stem cells can be metastasized to another organ and lead to the formation of new cancer. The treatment with CSC specific therapy can eradicate all the CSC population. The other cancer cells can be destroyed by immune system or conventional therapies. (B) Treatment with CSC specific therapy can kill all the CSC cells. The rest of the cancer mass can be eradicated with conventional chemo/radiotherapy. (C) Treatment with conventional therapy cannot destroy the CSC population due to their resistance mechanism and relative quiescence state. This may lead to the formation of new cancer. A combined approach including CSC specific therapy as well as conventional therapy could fully eradicate the cancer.

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Table-1: Colorectal cancer stem cells' biomarkers

Protein markers	**Gene**	**Assay method**	**References**
Nanog, Oct-4, SOX-2	*Nanog, POU5F1,SOX-2,*	Therapy resistant assay; quantitative RT-PCR	20-24, 33
CD133	*PROM1*	Chemoresistance assay; colony formation assay	9,15, 34, 99- 101
Lgr-5	*LGR5*	Tumorigenicity assay; experimental metastasis assay	40,48
CD24	*CD24*	Colony formation assay; invasion assay; differentiation assay; survival assay.	48, 85, 86
CD29	*ITGB1*	Colony formation assay	48
ALDH-1	*ALDH1A1*	Xenotransplantation in immunodeficient mice	52, 73
EpCAM	*EPCAM*	Immunohistochemistry;Western blot assay	59, 99
CD44	*CD44*	Xenotransplantation in immunodeficient mice; colony formation assay	48, 59, 89, 139
CD166	*ALCAM*	Tumour growth in immunodeficient mice following xenograft; colony formation assay	48
CD26	*DPP4*	Tumour formation and metastasis following xenotransplantation	70

Table-2: Cancer stem cell markers for the prognosis of colorectal cancer

Name of Marker	Expression in normal or non-cancer stem cells	Function	Role in prognosis of colorectal cancer	References
ALDH1	Several tissues and highest in the liver	Detoxifying enzyme and responsible for oxidation of intracellular aldehydes	Overexpression is associated with cancer release, distant metastasis , higher cancer grade and poor patients' survival	54, 72-74
CD24	B-lymphocytes and differentiating neuroblast	Cell adhesion molecule	Increased expression is correlated with poor patients' survival	83, 85, 87
CD44	Epithelial cells	Cell surface glycoprotein and involved in cell adhesion and migration, participate in malignant progression (adenoma to carcinoma)	Decreased or loss of expression is correlated with poor patients' survival	54, 86, 89, 91, 92
CD133	Stem cells in different organs	Regulation of stemness, associated with primitive cells and transmembrane glycoprotein	Elevated expression at protein and mRNA level is associated with poor patients' survival	35, 42, 98-100,
CD166	Activated T cells, fibroblasts, neurons, activated monocytes and melanoma cells.	Cell adhesion molecule, involved in neuronal extension, embryonic haematopoiesis, embryonic angiogenesis and associated in the development of adenoma to carcinoma.	Irregular and over expression is associated with shortened patients' survival.	54, 110
EpCAM	Epithelial tissue, progenitor cells and stem cells	Cell adhesion, participate in Cadherin-Catenin and Wnt pathway	Reduced expression is associated with lymph node metastasis, infiltrating tumour margin, higher cancer grade, vascular invasion, distant metastasis and poor patients' survival	54, 56, 114, 118
Lgr-5	Adult stem cells, muscle, placenta, spinal cord and brain	Associated with intestinal stem cells and downstream target of Wnt pathway	Higher expression is associated with lymph node metastasis, distant metastasis and poor patients' survival	35,37, 120-122, 124,
Nanog	Embryonic stem cells and epithelial cells	Transcriptional regulator, self-renewal	Elevated expression is associated with lymph node metastasis and poor patients' survival	125, 126
SOX-2	Embryonic stem cells, neuronal cells in the stomach and central nervous system	Transcription factor and regulates self-renewal or pluripotency of undifferentiated.	Overexpression is correlated with recurrence and lower disease free survival.	35
Oct 4	Stem cells in different organs	Regulation of stemness.	Expression is negatively correlated with cancer depth, lymph node metastasis and lymphatic invasion	23