Novel therapeutics against breast cancer stem cells by targeting surface markers and signaling pathways

Running title : Therapeutics against breast cancer stem cells

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Abstract

Background: Breast cancer remains to be one of the deadliest forms of cancers, owing to the drug resistance and tumor relapse caused by breast cancer stem cells (BCSCs) despite notable advancements in radio-chemotherapies.

Objectives: To find out novel therapeutics against breast cancer stem cells by aiming surface markers and signaling pathways.

Methods: A systematic literature search was conducted through various electronic databases including, Pubmed, Scopus, Google scholars using the keywords "BCSCs, surface markers, signaling pathways and therapeutic options against breast cancer stem cell. Articles selected for the purpose of this review, were reviewed and extensively analyzed.

Results: Novel therapeutic strategies include targeting BCSCs surface markers and aberrantly activated signaling pathways or targeting their components, which play critical roles in self-renewal and defense, have been shown to be significantly effective against breast cancer. In this review we represent a number of ways against BCSCs surface markers and hyper-activated signaling pathways to target this highly malicious entity of breast cancer more effectively in order to make a feasible and useful strategy for successful breast cancer treatment. In addition, we discuss some characteristics of BCSCs in disease progression and therapy resistance.

Conclusion: BCSCs involved in cancer pathogenesis, therapy resistance and cancer recurrence. Thus, it is suggested that a multi-dimensional therapeutic approach by targeting surface markers and aberrantly activated signaling pathways of BCSCs alone or in combination with each other could really be worthwhile in the treatment of breast cancer.

Keywords: Breast cancer, BCSCs, surface markers, signaling pathways, therapeutic options.

1. Introduction

Breast cancer is the deadliest form of cancer, accounts for 30% of all cancers diagnosed in women [1]. It causes more than half a million deaths per year worldwide [1]. The mortality rate of the disease is expected to rise 20% by 2020, despite recent advancement in treatment [2-3]. Extreme heterogeneities of this disease limit the effectiveness of conventional therapies, thereby approximately 40% of patients diagnosed with benign breast cancer advance to malignancies and causes disease recurrence despite having chemo-radiotherapies [4-5]. In addition, approximately 70% of them experience cancer relapse in less than five years [5]. Although, in recent years, treatment options of breast cancer have been improved explicitly, therapy resistance and side effects aggravated the necessity to search new therapeutic options to bring down resistance and to minimize the adverse side effects in patients with breast cancer [6].

Recent studies demonstrated that cancer stem cells (CSCs), a small subpopulation of cancer cells, are responsible for cancer initiation, progression, metastatic spread, therapy resistance and cancer recurrence, thereby contributing in making the diseases incurable [7-8]. In breast cancer, the main barrier to make a way through therapeutic resistance is the presence of breast cancer stem cells (BCSCs) [7]. Since BCSCs have more therapy resistant properties than bulk tumor cells, this population of cells should be the target for developing new therapies [7]. Therefore, targeting BCSCs could improve the efficacy for the treatment of breast cancer, at the same time abrogating the incidence of therapy resistance and cancer recurrence [8].

A numbers of studies reported effective anticancer potency against breast cancer cells by targeting BCSCs subpopulation [9-13]. For example, targeting BCSCs markers can inhibit growth and proliferation of BCSCs and could be an attractive option to treat breast cancer [10]. In addition, inhibiting overactivation of signaling pathways in CSCs by various methods

has important clinical implication in breast cancer [11-13]. Considering the potentials of these approaches in eradicating BCSCs as well as breast cancer cells, we have illustrated how BCSCs could be targeted to minimize their growth and proliferation, thereby, improve therapy resistance and disease recurrence. In addition, basic features of BCSCs associated with self-renewal and therapy resistance have been outlined.

2. Breast cancer stem cell (BCSCs)

Breast cancer is an extremely heterogeneous disease in nature and breast cancer stem cell (BCSC) is a small subpopulation of the breast tumor [14]. They can originate from stem cells, progenitor cells or differentiated cells through genetic and epigenetic alteration [15]. Although, the volume of this subpopulation in tumors is very low, ranging from 0.1-5% of total cell population and was first identified in 2003 by Al hajj *et al.* [16]. It was demonstrated that as few as a hundred of CD44 (+)/CD (-) cells from primary breast tumor were able to reproduce the tumor growth when injected into mammary fad pads immunocompromised mice [16]. Whereas tens of thousands of alternate phenotypic cells unable to form the tumor in recipient's mice [16]. Subsequently, BCSCs are characterized and well defined with the expression of different type of stemness markers, including cluster of differentiation 44 (CD44), cluster of differentiation 24 (CD24), cluster of differentiation 133 (CD133), epithelial cell adhesion molecule (EpCAM), cluster of differentiation 49f (CD49f), aldehyde dehydrogenase 1 (ALDH1), ATP binding cassette 2 (ABCG2) etc. [17-20]. These markers of BCSCs are associated with many cellular functions, including cellular adhesion, proliferation, differentiation and survival [21]. Most importantly, BCSCs utilizes multiple lines of self-defense mechanisms against therapeutic insults, thereby contributing in therapy resistance [22-23]. For example, over activation of ATP-binding cassette (ABC) transporter system in BCSCs enables them to pump out chemotherapeutic drugs [22]. In addition, they have active

DNA repair system, which can help them to repair their DNA immediately after chemo-radiotherapy, resulting in escaping DNA-damage induced apoptosis [23]. Moreover, signaling pathways *e.i.* Notch, Wnt/Frizzled/ β -catenin, Hedgehog and Hippo signaling are highly regulated in normal stem cells, whereas in BCSCs, they are aberrantly active, lead to uncontrolled growth and proliferation [24-25]. Furthermore, despite BCSCs exhibit features of stem/progenitor cells, there is plasticity which allows the acquisition of stemness traits by bulk tumor cells as well [26]. Thus, phenotypic and functional heterogeneity arise among cancer cells as a result of genetic, epigenetic changes and by environmental stimuli.

3. BCSCs in therapy resistance

Therapy resistance is the main obstacle of cancer treatment and current evidence suggests that this is largely contributed by the presence of therapy resistant CSCs, which are endowed with numerous resistant mechanisms to conventional therapies [27, 29-30]. In spite of intensive studies in recent time, the mechanisms by which BCSCs become resistant to therapies are not fully understood [28]. However, several principles have been proposed, conferring BCSCs' enhance resistant properties against drugs, ionizing radiation, and cell stress [27]. In BCSCs, four vital mechanisms are associated with therapy resistance. Firstly, BCSCs have a prominently activated ATP binding cassette (ABC) or drug efflux pump, which can successfully pumps out chemotherapeutic drugs such as anthracycline or taxanes (two key drugs of breast cancer treatment) [29-30]. Secondly, Aldehyde dehydrogenase 1 (ALDH1) is highly active in BCSCs which is a key enzyme to oxidize intracellular aldehyde to carboxylic acid, thereby removing toxic oxygen radicals from the tumor microenvironment [31]. Thirdly, BCSCs possess highly active DNA repair system, which repairs DNA damages particularly after treatment with chemo-radiotherapies [32]. Finally, BCSCs are inherently safe from numbers of chemotherapeutic drugs which target cell cycle process. They protect

themselves from these drugs by maintaining a quiescent state in G₀ phase of the cell cycle [33]. In addition, a number of enzymes such as superoxide dismutase, catalase and glutathione peroxidase are highly expressed in BCSCs, which prevent them from genotoxic insults of reactive oxygen species (ROS) [34-35]. Besides, BCSCs are mostly located in hypoxic areas, which also confer more protective niche for them against chemo-radiotherapies. Hypoxia increases the BCSC population through hypoxia-inducible factors (HIF-1 and HIF-2) that prompt cell dedifferentiation by upregulation of embryonic stem cell markers [36]. In different breast cancer cell lines *e. g.* MDA-MB-231, SUM-149 and SUM-159 *etc.* upregulation of HIF and transcription factor such as MDR1 results in BCSC population enrichment through interleukin-6 (IL-6) and IL-8 signaling and multi drug resistant 1 (MDR1) overexpression [37]. Other mechanisms include activation of PI3K/Akt signaling via phosphatase and tensin homolog (PTEN) driving cell cycle arrest, activation of Wnt/ β -catenin, Hh and Notch signaling and constitutive activation of nuclear factor kappa beta (NF- κ B) [38-41].

4. Targeting surface markers of BCSCs

Despite the abundance of CSC surface markers on BCSCs, it is difficult to target majority of them because they are also expressed in normal stem cells [42]. However, therapies targeting BCSCs with specific phenotypic surface markers open a sensible horizon for potential therapies against breast cancer [42-48]. Accordingly, a numbers of drugs have already been in testing, which can act on specific biomarkers of BCSCs, thereby could eradicate BCSCs [42-48] (Table 1). For example, CD44 is the most commonly used BCSCs marker, and development of antibody against CD44, which in turn could induce terminal differentiation of BCSCs has already been found effective [42]. Extracellular matrix components act as ligand for all CD44 variants and accumulating evidence suggests that

these interactions regulate many signaling pathways, which play indispensable role in tumor progression [43]. Thus, antibodies developed against highly expressed CD44 variants have the potential to inhibit and disrupt CD44-matrix interactions, thereby halt tumor growth and progression (Table 1) [44]. There are two main ways by which CD44 can be targeted [45]. Firstly, employing native antibody to bind and resist receptor by competitive inhibition of its ligand (extracellular matrix components) as a result preventing receptor signaling cascade (Figure 1). Secondly, chemotherapeutic agents, toxins or radioisotopes can be attached with antibodies to induce cell death (Figure 1) [45]. Moreover, antibodies attached with nanoparticles are gaining attention now-a-days [46]. For example, anti-CD44 antibodies-conjugated with gold nano-rods and tumor sensitizing drug (paclitaxel) have been used to sensitize MCF-7 breast cancer that overexpresses the CD44 surface marker [46]. Disruption of extracellular matrix component hyaluronan (HA)-CD44 -interaction by using HA oligomers is another approach to target CD44 [45]. This approach involves replacing the multivalent interaction of high molecular weight HA and CD44 with monovalent interaction of small oligomers of HA (6–18 saccharide units of HA) [45]. It was observed that HA-oligosaccharides suppress bone metastasis in breast cancer via intercepting HA-CD44 interaction [43]. Moreover, according to some studies, small HA oligomers have shown their potential during in vivo treatments by suppressing tumor growth and inducing tumor regression in xenografts models of several tumor types [43, 47-51]. Furthermore, enzymatic and non-enzymatic degradation of HA could be used as a useful therapeutic tool against breast cancer. Hyaluronidases, a class of enzymes, have the potential to minimize breast cancer progression by the degradation of HA as over expression of hyaluronidases is correlated with breast cancer progression. Consistently, in vitro knockdown of hyal1 gene (encode hyaluronidases) in MCF7 and ZR-75-30 cells exhibited reduced cell growth and invasion, while overexpression of the isoenzyme caused elevated cell malignancy [52].

Furthermore, in vivo study using MCF7-cells demonstrated that induced hyal1 overexpression resulted in increased tumor growth and promoted angiogenesis in a nude mouse model [52]. Thus targeting CD44 by intercepting the interactions of CD44 with extracellular matrix component could bring a therapeutic solution against BCSCs.

CD133 also known as prominin, another BCSC marker, is critical for BCSCs growth, proliferation and survival [53]. CD133 upregulation was observed in tumor initiating or BCSCs and it was suggested that immunotoxin/antibody developed against CD133 could be useful to inhibit BCSCs growth [54]. Before testing effectiveness of anti-CD133 antibody against breast cancer, a fusion protein dCD133KDEL, resulting by fusing a gene fragment encoding the scFv portion of an anti-CD133 antibody to a gene fragment encoding deimmunized PE38KDEL, exhibited potent antitumor activity following intratumoral delivery into head neck cell carcinoma xenografts [54]. Not surprisingly, in breast cancer, it was found that dCD133KDEL selectively inhibits MDA-MB-231 ductal breast carcinoma cells containing CD133 (+) subpopulation. Furthermore, in a model of metastatic breast cancer, systemic administration of dCD133KDEL caused regression of tumor growth. Moreover, combined therapy of dCD133KDEL with an immunotoxin designed to target the bulk tumor mass was noted highly effective therapeutic, suggesting dCD133KDEL as a promising therapeutic tool against breast cancer cell expressing CD133+. In addition, a commercially available monoclonal anti-CD133 antibody, namely AC-133 was tested against breast cancer in combination with saponin (a highly used toxin) [55]. It was found that combination of AC-133 with saporin (AC133- saporin) causes arrest cell proliferation of CD133+ subpopulation [55].

Studies using in vivo and in vitro model suggest that compounds of natural origin have the potential to reduce the expression of BCSCs markers, thereby could inhibit growth and proliferation (Table 1) [56-61]. For example, β -lapachone (bL), an o-naphthoquinone,

has many pharmacological properties including inhibitory effects against several cancers such as prostate, colon, ovarian, lung and breast cancer [56-61]. In MDA-MB-231 cells bL inhibits the expression of BCSCs markers, *e. g.* ALDH1 and CD44 in a NQO1 (nicotinamide adenine dinucleotide phosphate (NAD(P)H): quinoneoxidoreductase) dependent manner [62]. NQO1 is an antioxidant enzyme and its expression level is highly enhanced in various cancers including breast cancer [63]. Treatment of BCSCs with bL inhibited the proliferative ability of mammospheres, derived from BCSCs in a NQO1-dependent manner [62]. In addition, down regulation of BCSCs markers such as CD44, aldehyde dehydrogenase 1 family member A1 (ALDH1A1), and discs large (DLG)-associated protein 5 (DLGAP5) was occurred in both cultured cells and mammosphere cells followed by bL treatment.

Doxycycline (antibiotic), an inhibitor of mitochondrial biogenesis, suppressed the expression of stemness markers CD44 and ALDH1 after oral supplementation in patients with breast cancer [64]. It was noted that tissue samples of orally treated patients showed 40% reduction of CD44 expression when compared to that of untreated controls. Moreover, more than 50% reduction was noted in 4 out of 9 patients. In addition, the expression of ALDH1 was reduced as much as 60-90% after receiving oral doxycycline supplementation in comparison to that of non-treated controls [64]. Consistent with this finding, an *in vitro* analysis also showed significant reductions in the CD44 (+) / CD24 (-/low) CSC population in MCF7 and MDA-MB-468 cell lines after doxycycline treatment [65]. Likewise, the expression levels of other “stemness” markers (Oct4, Sox2, Nanog and CD44) were also reduced by >50%, in response to doxycycline, as assessed by mRNA levels and independently confirmed by immuno-blot analysis. Moreover, doxycycline has been shown to reduce ALDH (+) breast CSCs in HER2 (+) and triple-negative human breast cancer cells [66].

Another marker, nucleostemin might be an attractive option to target self-renewal and proliferation of BCSCs for better management of patients with breast cancer [67].

Interestingly, indole-3-carbinol (I3C), a natural compound, induces anti-cancer effect against BCSCs in MCF-7 and MDA-MB-231 cells by targeting nucleostemin [67]. I3C inhibits the interaction between p53 tumor suppressor protein and murine double mutant-2 (MDM2) [67]. As an inhibitor, MDM2 interacts with p53 through a signaling cascade, thereby inhibiting p53 to trigger its apoptotic response [68]. However, I3C treatment promotes the interaction of nucleostemin with murine double mutant 2, resulting in disruption of the interaction between MDM2 and p53 [67]. In addition, I3C induced nucleostemin to sequester MDM2 in a nucleolus compartment, as a result freeing p53 to mediate its apoptotic activity [67]. Furthermore, knockdown of nucleostemin by small interfering RNA functionally proved that nucleostemin is required for I3C to trigger its cellular anti-proliferative responses, to inhibit tumorsphere formation, and also to disrupt MDM2–p53 protein–protein interactions [67]. Moreover, the expression of I3C-resistant form of an elastase, only known target protein for I3C, prevented I3C anti-proliferative responses in cells and in tumor xenografts in vivo, as well as disrupted the I3C-stimulated nucleostemin–MDM2 interactions [67].

Quercetin is a well-known natural compound inhibits MDA-MB-231 CSCs by downregulation of ALDH1A1, chemokine receptor type 4 (CXCR4), epithelial mesenchymal adhesion molecule (EpCAM), and mucin 1 (MUC1) [69]. It has been reported that ALDH1A1, CXCR4, EpCAM, and MUC1 may be potential targets for breast CSC treatment [69]. Quercetin treatment showed significantly lower ALDH1A1, CXCR4, EpCAM, and MUC1 levels compared to those of the control group. Moreover, quercetin-induced suppression of these stem cell markers resulted in inhibition of mammosphere formation and migration of CSCs [69].

It is well established that estrogen regulates the physiological processes of normal breast cells, and breast cancer cells depending on the presence of receptor (ER) [70-71]. ER α is frequently over-expressed in ER-positive breast cancer cells, and subsequently regulates the cell cycle in these cells, suggesting that E2-ER α signaling plays a critical role in cell proliferation [72]. Treatment BCSCs with 17 β -estradiol impedes migration, self-renewal and colony formation ability of BCSCs by modulating the expression of Sox-2 [72]. The decreased maintenance of stemness of CSCs inspired to evaluate the expressional changes of Oct4, Sox2, Klf4 and c-Myc, which are vital for stemness, and it was revealed that the Sox2 mRNA level was significantly decreased by 50 nM 17 β -estradiol treatment [72]. Therefore, estrogen treatment may inhibit malignancy of BCSCs by downregulating the expression of Sox2.

A glycosphingolipid, GD2 is another cell surface marker of BCSCs. It has been observed that GD2⁺ breast cancer cells (derived from cell line or clinical sample) show stemness properties [73]. However, GD3 synthase, the rate-limiting enzyme for biosynthesis of GD2, can be utilized as a therapeutic target for the management of BCSC phenotype. GD3 synthase (GD3S) is highly expressed in GD2⁺ as well as in CD44⁺CD24⁻ cells. Therefore, targeting GD3 synthase either by shRNA or small molecule triptolide reduces the CSC enrichment and diminishes the formation of primary tumor [73]. Moreover, GD3S knockdown completely abrogated tumor formation in vivo. In addition, genetically engineered anti-GD2 antibodies or fragments have been tested for effective management of patients with various cancers [74].

Unlike other conventional surface markers of BCSCs, Aldehyde dehydrogenase 1 (ALDH1 is an enzyme, which is positively correlated to BCSCs by its functional role in maintaining stemness [75]. Thus targeting ALDH1 can be a useful therapeutic intervention to eradicate BCSCs. Withaferin A (a natural steroidal lactone) has been reported to target

expression of ALDH1 and subsequently causing BCSCs to abrogate its stemness [75].

Withaferin A reportedly inhibited in vitro mammosphere formation along with the inhibition of Oct4, SOX-2, and Nanog expression. Most importantly, Withaferin A administration to MMTV-neu mice resulted in inhibition of mammosphere number and ALDH1 activity [75]. Additionally, ALDH1+ BCSCs can be targeted and eliminated by photothermal therapy (PTT)-mediated highly crystallized iron oxide nanoparticles [76]. PTT is one of the few nanoparticle based treatments that enters clinical trial to induce localized hyperthermia in human cancer patients [76]. Recently, it has gained attention for its ability to induce a systemic immune response targeting distal cancer cells in mouse models [76]. Iron oxide nanoparticles mediated PTT effectively kills BCSCs in translational models of triple negative breast cancer. In addition, PTT inhibits BCSC self-renewal through reduction of mammosphere formation in primary and secondary generations. Secondary implantation confirmed that PTT significantly inhibits ALDH + BCSCs in NOD/SCID mice [76]. Thus, aiming surface markers to inhibit their functions in BCSCs could provide a successful way to eradicate tumor growth and progression.

5. Targeting signaling pathways of breast cancer stem cell

Growth and development of normal cells are highly maintained by different signaling pathways and dysregulation of these pathways resulting in different pathophysiological diseases [77-78]. Accordingly, dysregulation of these pathways in BCSCs is involved in tumor formation and progression [79]. For example, deregulation of pathways such as Notch, Wnt/ β -catenin, hedgehog, hippo signaling *etc.* are involved in cancer formation and progression [80-82].

Hyperactivation of Notch signaling is correlated with various cancers, including breast cancer [83-86]. In normal epithelial cells, activation of Notch signaling is tightly

regulated by active Notch receptors. However, uncontrolled activation of these receptors is associated with the induction of breast tumor formation [87]. In addition, Notch signaling is associated with therapy resistance, therefore, has become a subject of absolute interest in breast cancer research [88]. Notch signaling comprised of five Notch ligands and four receptors, which become activated upon ligand binding [89]. This interaction causes the release of Notch intracellular domain, which in turn, acts as a transcription factor, thereby regulating transcription of downstream target genes (Figure 2) [89]. It was reported that over activation of Notch receptors in BCSCs is associated with poor prognosis of patients with breast cancer [90]. Interestingly, reduction of γ -secretase activity significantly decreased Notch signaling and subsequently inhibited mammosphere formation capacity of BCSCs [91]. MK-0752, a γ -secretase inhibitor, in combination with docetaxel, has reached phase I/II clinical trials (NCT00645333) for metastatic breast cancer. The benefits of BCSCs targeted therapy, specially the inhibition of Notch pathway in combination with systemic cytotoxic drugs, was evidenced from biopsies of patients showing a decrease of cell population with CD44⁺, CD24⁻ phenotype, and ALDH⁺ activity [91]. A protein-protein interaction inhibitor CB-103, targets Notch signaling, is currently in phase I/II clinical trial (NCT03422679) for advanced or metastatic breast cancer [7]. In addition, psoralidin, a plant-based inhibitor of Notch signaling has been shown to effectively decrease bulk tumor size, upregulates pro-apoptotic genes, and inhibits BCSCs proliferation and self-renewal [92]. Moreover, Vitamin D compounds such as 1 α , 25 (OH) 2D3 and Gemini analog of vitamin D, BXL0124 have been found effective in regulating BCSC differentiation and reduction by inhibiting Notch pathway. The vitamin D compounds show the activity against BCSCs by impeding the expression of Notch signaling components such as Notch1, Notch2, Notch3, JAG1 and JAG2 [93]. Therefore, targeting Notch receptors, ligands and other components of Notch signaling

by antibody, natural and synthetic compounds may provide a fruitful solution in better management of patients with breast cancer.

The Wnt signaling is an essential regulator of mammary development and over-activation of this pathway is highly correlated with breast tumorigenesis [94]. β -catenin is a downstream component in the canonical Wnt pathway, which acts as a transcription activator and involved in determining BCSCs migration, polarity and differentiation [95]. The Wnt proteins along with Frizzled receptors and Dishevelled phosphoprotein regulate β -catenin degradation [96]. Down regulation or inactivation of these components leads abnormal accumulation of β -catenin (Figure 2), resulting in increased growth and proliferation of BCSCs [96]. It has been reported that approximately 50% cases of breast cancer show elevated level of stabilized β -catenin, whereas Dishevelled is amplified in 50% breast cancers [97-98]. Therefore, aiming at the uncontrolled activation of these important regulators could provide better therapeutic options against breast cancer. Moreover, in non-canonical or β -catenin independent pathway Wnt ligand stimulates the planar cell polarity (Wnt/JNK) and the Wnt/ Ca^{2+} pathway [99]. Recent studies reported potential therapeutics notably kill cancer cells, especially BCSCs by targeting canonical and non-canonical Wnt signaling [100-103]. A numbers of natural compounds such as curcumin, ellagic acid, koenimbin and Sulforaphane etc have been found effective to inhibit uncontrolled activation of canonical Wnt signaling in BCSCs (Figure 2) (Table 2) [100-103]. For instance, curcumin, a polyphenolic curcuminoid isolated from turmeric, coupled with mitomycin C (an anticancer agent) inhibits the growth of MCF-7 BCSCs via Wnt/ β -catenin pathway [100]. Combined treatment of curcumin and mitomycin C decreased the expression of β -catenin and induced apoptosis of BCSCs [100]. Another naturally occurring compound ellagic acid directly inhibits ACTN4 and limits the metastasis of breast cancer stem cells via regulating Wnt/ β -catenin signaling [101]. ACTN4 is a critical factor in breast cancer cells' metastasis [101]. It

was noted that over expression of ACTN4 is observed in several basal-like breast cancer cell lines (MDA-MB-231, BT-549, HCC 1937) when compared to non-neoplastic mammary cell line (MCF-10A). ACTN4 facilitates BCSCs self-renewal by stabilization and accumulation of β -catenin. However, treatment with ellagic acid reduced ACTN4 expression, subsequently inhibited β -catenin to exhibit its downstream action [101]. Koenimbin, a naturally occurring dietary compound also decreases mammosphere formation of CSCs in MCF-7 cell line [102]. Koenimbin treatment resulted in decreased cell population in MCF7 stem cells and also significantly inhibited the size and number of MCF7 stem cells in primary, secondary, and tertiary mammospheres in vitro. In addition, koenimbin increased β -catenin phosphorylation and degradation by glycogen synthase kinase 3 β [102]. Sulforaphane, another natural compound of broccoli, eliminates BCSCs by modulation of Wnt/ β -catenin pathway [103]. Sulforaphane mainly targets β -catenin expression in translational level and increases proteasome mediated degradation of β -catenin [103]. Sulforaphane decreased aldehyde dehydrogenase-positive cell population by 65% to 80% in human breast cancer cells. It also reduced the size and number of primary mammospheres by 8- to 125-fold and 45% to 75% respectively [103]. A Schiff base, monobenzyltin complex C1 showed similar kind of activities against BCSCs in MCF-7 cell line [104]. Monobenzyltin complex C1 showed a significant reduction in the aldehyde dehydrogenase-positive cell population and a significant reduction in the population of MCF-7 cancer stem cells in primary, secondary, and tertiary mammospheres. Moreover, C1 treatment reported to down-regulate the Wnt/ β -catenin self-renewal pathway in BCSCs [104]. Furthermore, celecoxib, a non-steroidal anti-inflammatory drug (NSAID), has been found to be selectively targeting self-renewal, EMT, metastasis and tumorigenesis abilities of BCSC in vivo [105]. Celecoxib sensitizes MCF-7 and MDA-MB-231 cells to 5-FU and cisplatin by effectively targeting CSCs. It was reported that, celecoxib exhibits its actions by inhibiting prostaglandin E2 (PGE2) synthesis and

downregulating Wnt signaling. PGE2 is a strong inducer of inflammation. It was noted that celecoxib downregulates the Wnt pathway by inhibiting the formation of PGE2 and by decreasing the phosphorylation of GSK-3 β [105]. Furthermore, it was found that celecoxib decreases mRNA expression of Wnt target genes such as Cyclin D1, Axin 2 and c-Myc [105]. A clinical trial (NCT01351103) of LGK-974 (WNT974) alone or in combination with immunotherapy (an anti-PD-1) recruits patients with breast cancer. LGK-974 is an inhibitor of the endogenous Wnt palmitoleoylase PORCN which is required for the palmitoylation of Wnt ligands, a necessary step in the processing of Wnt ligand secretion [106]. It was found that LGK-974 significantly inhibits Wnt signaling in vitro and in vivo, including reduction of the Wnt-dependent LRP6 phosphorylation and the expression of Wnt target genes, such as AXIN2 [106]. Another clinical trial (NCT02655952) has been started using a formylated six amino acid peptide fragment (Foxy-5) that mimics the effects of Wnt5a. Wnt5a is a non-canonical member of the Wnt family, which plays an important role in organ development, cell polarity, and migration, therefore, acting as an anti-metastatic cancer drug [7]. Therefore, blocking Wnt signaling by inhibitor or targeting β -catenin expression or facilitating β -catenin degradation by activating GSK3 β could drastically reduce the action of Wnt/ β -catenin signaling in BCSCs, thereby inhibiting the growth and proliferation of breast cancer cells.

Hedgehog signaling is another crucial regulator of proliferation, maintenance and self-renewal ability of BCSCs [107]. Components of Hedgehog signaling have been associated with activation of BCSCs. Binding of Hedgehog ligand with Patched receptor leads to the release of Gli family proteins and causes subsequent transcription of target genes [108]. A well-known Hedgehog antagonist cyclopamine has been shown to decrease BCSC proliferation and ultimately inhibits tumor growth in breast cancer [109]. Cyclopamine, the first Hh inhibitor to be identified, inhibits the Hh pathway by binding to and inactivating the Smoothened (SMO) transmembrane receptor protein [109]. Cyclopamine suppressed

expression of Gli and the growth of the Hh pathway-activated breast cancer [109]. Nitidine chloride, another naturally occurring compound, inhibits CSCs like properties of MDA-MB-468 and MCF-7 cell lines by intercepting Hedgehog signaling pathway [110]. The activity of nitidine chloride (NC) was confirmed by standard Hedgehog inhibitor. Suppression of Hedgehog pathway by NC directly reverses epithelial mesenchymal transition as well as inhibits the expression of CD44 and subsequently abrogates BCSC properties [110]. Another study suggested that co-treatment of breast cancer cells with Hh inhibitor and docetaxel decreased CD44⁺/CD24⁻ BCSCs population and mammosphere formation. On the contrary, both of these properties increased when treated with docetaxel alone [111]. They found a link between the activation of Hh signaling and over expression of MDR1 and ABCG2 in BCSCs and thus providing evidence for the inhibition of this pathway to avoid resistance to first-line therapy [111]. Only two Hh inhibitors have made their way to clinical trial in the face of several promising pre-clinical results [112-113]. GDC-0449 (vismodegib) with paclitaxel, epirubicin and cyclophosphamide (NCT02694224) and LDE225 (sonidegib) combined with Docetaxel (NCT02027376) [112-113]. Both of the drugs were tested in triple negative breast cancer. Inhibiting collagen remodelling and cancer stem cell plasticity has the potential to reduce chemoresistance against docetaxel in triple negative breast cancer [113]. Hedgehog ligand produced by cancer cells provides favorable microenvironment for the acquisition of CSCs properties by reprogramming cancer associated fibroblasts (CAFs). Reprogrammed CAFs facilitates the favorable condition for CSCs by expression of fibroblast growth factor 5 (FGF5) and by the production of fibrillar collagen [113]. Interestingly, treatment of breast cancer with Hedgehog receptor inhibitor (SMOi) sonidegib causes reduced expression of CSCs markers and sensitizes cancer cells towards docetaxel, thereby improving survival of patients with cancer [113]. In phase I clinical trial, 3 of 12 patients with metastatic TNBC benefited from combination therapy of Sonidegib and docetaxel, with one patient

experiencing a complete outcome. These studies recognize Hh signaling as a novel mediator of CSC plasticity and a promising therapeutic target in TNBC [113]. Thus, there is no denying the fact that, inhibiting Hedgehog receptor, ligand or transcription activator Gli protein can reduce the overactivation of this pathway and halts the abnormal growth of BCSCs.

Hippo signaling is a major regulator to maintain organ size, stem cell renewal and tumorigenesis [114-115]. It is regulated by a complicated network of core kinase cascade, consisting of Mst1/2 and Lats1/2 [114]. The transcription of Hippo target genes is regulated by phosphorylation of Yes associated protein 1 (YAP1) and TAZ by Mst1/2 and Lats1/2 (Figure 2) [115]. However, dephosphorylation leads to nuclear translocation of YAP1 and TAZ, resulting in transcription activation. Studies suggested that in metastatic breast cancer, overabundance of YAP and TAZ is occurred in CSCs [116]. Therefore, targeting this pathway component may impose a way to inhibit the hyper activation of Hippo signaling. Interestingly, apigenin, a naturally occurring compound, can attenuate migration of CSCs in triple negative breast cancer cell by inhibiting transcription activities of YAP1 and TAZ [117]. Furthermore, apigenin robustly inhibited the features of stemness in triple negative breast cancer cells (MDA-MB-436 and MDA-MB-231), evidenced by a decrease in the CD44⁺ /CD24⁻ CSC subpopulation and mammosphere formation [117]. Besides, limiting dilution analysis of tumorigenesis confirmed that apigenin inhibits the tumor-initiating properties of triple negative breast cancer cells in vivo. Moreover, apigenin significantly decreased the expression of CTGF and CYR61, two YAP/TAZ-regulated genes, at both the mRNA and protein level [117]. It was also evaluated that, apigenin disrupts the interaction of TAZ to TEAD which is vital for maintaining CSCs trait in MDA-MB-436 triple negative breast cancer [117]. These results suggest that apigenin might offer a novel therapeutic option for triple negative breast cancer cells patients with high YAP/TAZ activity.

Signal transducer and activator of transcription 3 (STAT3) is also a key signaling pathway in BCSCs and induces the expression of genes including ALDH1 [118]. STAT3 is activated by phosphorylation [91]. In BCSCs, inhibition of STAT3 by glucosamine treatment (inhibitor of STAT3 phosphorylation) caused downregulation of ALDH1 expression [118]. It was determined that glucosamine treatment decreases the viability of ALDH⁺ breast CSCs. It also inhibited the stemness of ALDH⁺ breast CSCs, as indicated by decreased ALDH1A1, OCT-4 and KLF4 expression level, and a decreased number of mammosphere formation. This effect of glucosamine may be associated with a diminished pSTAT3/STAT3 ratio, indicating that glucosamine inhibits STAT3 activation [118]. Therefore, signaling pathways could be the critical points for developing molecular therapies targeting BCSCs. Hence, individually or combined therapies to target these pathways should hold better promise for eradicating breast cancer stem cells.

However, discovery and development of gene targets for the treatment of breast cancer will not be worthy if it does not focus on better clinical applications. It was noted that targeting specific genes by RNA interference or by retroviruses could facilitate the inhibition of BCSCs growth and proliferation [119]. In addition, with enhanced pathological understanding and biotechnological improvements, gene therapy will become a part of clinical practice against breast cancer [120]. Nevertheless, maximizing the potentials of gene therapy efficacy and sustained therapeutic gene (s) expression in target cells, low toxicity, and a high safety profile should be ensured [120].

6. Conclusion and future perspectives

Better outcome of patients with breast cancer could be possible if we defeat the challenges effectively and specifically, especially by targeting BCSCs. Therefore, herein we have attempted to take measure a representative review about how CSCs are responsible to

cancer pathogenesis and provided a bunch of useful ways to encounter BCSCs in order to make a feasible strategy for breast cancer treatment. To encounter BCSCs more effectively we should probably look into several strategies that hold future promise. They are as follows-

(i) Targeting BCSCs markers to intercept the interactions between surface markers and extracellular matrix components, which in turn might halt stemness property of BCSCs. Regarding this, a number of antibodies have been proposed to intercept these interactions and also in combination with standard radio/chemotherapy agents may be a promising future treatment for breast malignancy. (ii) Several pathways, including Notch, Wnt, Hedgehog, Hippo and STAT3 have been the crucial point for molecular therapies against BCSCs, and individual or combination therapies targeting these pathways hold future promise. However the molecular pathways that control the BCSC phenotype rarely operate individually. These pathways evolved to operate as a network during normal development, and they are linked by multiple redundancies and feedback mechanisms by forming a complex functional network. For example, GSK3 β , a downstream essential component of the Wnt pathway, is phosphorylated and inactivated by AKT, which is activated by Notch through multiple mechanisms. Hence, inhibition of one pathway may trigger compensatory feedback mechanisms that allow BCSC survival by up-regulation of other pathways. Therefore, rather than resolving a single “Achilles’ heel” of all BCSC that can be targeted by intercepting a single pathway, the functional networks that are responsible for the BCSC phenotype in breast cancer subtypes may have to be targeted through rational combinations. Finally, all of the presented targets discussed herein might be of use for the development of a BCSC-directed therapy, however, the plasticity of BCSCs to shift between stem-like and non-stem-like states implies that the targeted therapy must not be restricted to this small population, but rather to a combination treatment also addressing more differentiated progenitors and the bulk tumor cell population.

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Figure legends

Figure 1: Eradication of BCSCs by targeting surface markers. Ligands binding to the surface markers of cancer stem cells initiate downstream signaling cascade, which leads abnormal proliferation, invasion and therapies resistance. Using antibodies alone or in combination with chemotherapeutic drugs, immunotoxin or with radioisotopes could attenuate downstream signaling cascade which subsequently halt BCSCs growth and proliferation.

Figure 2: Targeting signaling pathways in BCSCs. Schematic representation of the Notch, Wnt, Hedgehog (Hh) and Hippo in BCSC. Novel therapeutics (synthetic and natural) kill BCSC by targeting these signaling pathways or their components.

Table1: Targeting surface markers in Breast cancer stem cells (BCSCs)

Compound/Drug /Technique	Mode of action	Target	Reference
HA (hyaluronan oligomer)	Suppression of tumor growth and induction of tumor regression	CD44	[45]
HA tagged nanocarriers	Nano-carriers carry known anticancer drugs. HA tagging increases efficiency of drug delivery due to CD44-HA interaction	CD44	[46]
CD44 siRNA	Makes BCSCs more sensitive to Doxorubicin	CD44	[121]
<i>hyal1</i> Knockdown	Degrades HA and subsequently inhibits BCSC growth and invasion	CD44	[52]
scFv- PE38KDEL	Induces cytotoxicity in BCSCs	CD133	[54]
AC133-saporin	Arrests cell proliferation	CD133	[55]
β -lapachone	Inhibits proliferative activity of BCSCs in a NQO1-dependent manner.	ALDH1, CD44, CD133	[62]
Doxycycline	Reduces BCSCs by induction of apoptosis	CD44, ALDH1	[64]
Indole-3-carbinol	Induces p53 mediated apoptosis	Nucleostemin	[67]
Quercetin	Downregulation of Sox2, Oct4, nanog and, Bmi-1	ALDH1, SCR4, EpCAM	[69]
17 β -estradiol	Impedes migration, self-renewal and colony formation ability of BCSCs	Sox-2	[72]
Triptolide	Targets GD3 synthase to inhibit BCSC and primary tumor formation	GD2	[73]
shRNA	Targets GD3 synthase to inhibit BCSC and primary tumor formation	GD2	[73]
Withaferin A	Downregulation of Sox2, Oct4, nanog and Bmi-1	ALDH	[75]

GD2:Disialoganglioside;**NQO1:** Nicotinamide adenine dinucleotide phosphate: quinoneoxidoreductase;HA:Hyaluronan;

Table2: Targeting signaling pathways in Breast cancer stem cells (BCSCs)

Compounds/Drugs	Mode of action	Targeted pathway	Reference
MK-0752	Sensitizes BCSCs to docetaxel	Notch	[91]
Psoralidin	Upregulates proapoptotic genes, and inhibits CSC proliferation and self-renewal.	Notch	[92]
1 α ,25(OH) ₂ D ₃	Inhibits Notch1, Notch2, Notch3, JAG1 and JAG2.	Notch	[93]
PF-03084014	Makes BCSCs vulnerable to known chemotherapeutic agents	Notch	[122]
Capsaicin	Prevents translocation of Notch intracellular membrane domain (NICD) into the nucleus	Notch	[123]
CB-103	Inhibits protein-protein interaction	Notch	[7]
Curcumin	Decreases β -catenin expression and induces apoptosis in combination with mitomycin C	Wnt/ β -catenin	[100]
Ellagic acid	Inhibits ACTN4 and limit the metastasis of BCSCs	Wnt/ β -catenin	[101]
Koenimbin,	Induces phosphorylation and degradation of β -catenin	Wnt/ β -catenin	[102]
Sulforaphane,	Increases proteasome mediated degradation of β -catenin	Wnt/ β -catenin	[103]
Monobenzyltin complex C1	Increases proteasome mediated degradation of β -catenin	Wnt/ β -catenin	[104]
Celecoxib	Inhibits the synthesis of prostaglandin E ₂ and down-regulating the Wnt pathway Activity	Wnt/ β -catenin	[105]
LGK974	Reduces phosphorylation of Wnt-dependent LRP6 and also inhibits the expression Wnt target genes	Wnt/ β -catenin	[106]
Foxy-5	Mimics the effect of Wnt5a and inhibits metastasis	Wnt/ β -catenin	[7]
Cyclopamine	Inactivates Smoothed (SMO) transmembrane receptor protein by direct binding	Hedgehog	[109]
Nitidine chloride	Reverses EMT as well as inhibits the expression of CD44 and abrogates CSC properties	Hedgehog	[110]
GDC-0449 (vismodegib)	Sensitizes breast cancer cells to paclitaxel, epirubicin and cyclophosphamide	Hedgehog	[112]
LDE225 (sonidegib)	Causes reduced expression of CSCs markers and sensitizes cancer cells towards docetaxel	Hedgehog	[113]
GANT61 (Gli protein inhibitor)	Downregulates effector molecules in the Hh pathway: glioma-associated oncogene	Hedgehog	[124]
Apigenin	Attenuates migration of BCSCs by inhibiting transcription activities of YAP1 and TAZ	Hippo	[117]
Glucosamine	Decreases viability and mammosphere formation of BCSCs	STAT 3	[118]