RESEARCH ARTICLE

Application of Stem Cells in Targeted Therapy of Breast Cancer: A Systematic Review

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Abstract

Background: The aim of this systematic review was to investigate whether stem cells could be effectively applied in targeted therapy of breast cancer. Material and Method: A systematic literature search was performed for original articles published from January 2007 until May 2012. Results: Nine studies met the inclusion criteria for phase I or II clinical trials, of which three used stem cells as vehicles, two trials used autologous hematopoetic stem cells and in four trials cancer stem cells were targeted. Mesenchymal stem cells (MSCs) were applied as cellular vehicles to transfer therapeutic agents. Cell therapy with MSC can successfully target resistant cancers. Cancer stem cells were selectively targeted via a proteasome-dependent suicide gene leading to tumor regression. Wnt/β-catenin signaling pathway has been also evidenced to be an attractive CSC-target. Conclusions: This systematic review focused on two different concepts of stem cells and breast cancer marking a turning point in the trials that applied stem cells as cellular vehicles for targeted delivery therapy as well as CSC-targeted therapies. Applying stem cells as targeted therapy could be an effective therapeutic approach for treatment of breast cancer in the clinic and in therapeutic marketing; however this needs to be confirmed with further clinical investigations.

Keywords: Breast cancer - stem cells - cellular vehicles - targeted therapy - systematic review

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Introduction

Breast cancer remains the most common malignancy among women worldwide, with an increase in incidence from 10.9 to approximately 20 million new cases per year by the year 2020, and a growing annual mortality from 6.6 to more than 10 million (Parkin et al., 2001; Roukos et al., 2007; Lehmann et al., 2011).

In spite of the clonal origin of tumors, increasing evidence in hematopoetic malignancies (Clarke et al., 2006) and many solid cancers suggest that the tumor cell populations are heterogeneous in terms of proliferation and differentiation (Massard et al., 2006). This feature could be well clarified by "cancer stem cell" hypothesis, and may answer to the ever increasing questions such as cancer progression and drug resistance. Cancer Stem Cells (CSCs) or cancer initiating cells (CICs) are a small population of cancer cells within tumors, which poses stem cell features like self-renewal, capability to develop multiple lineages, and capacity of proliferation (Heppner, 1984; Reya et al, 2001; Clarke et al., 2006; Dwyer et al., 2007; Bohl et al., 2011). Although the definite origin of CSCs is not completely identified yet, various possible origins for CSCs have been proposed including adult stem cells existing in many tissues, from a population of more differentiated transit amplifying/progenitor cells, embryonic stem cell-like cells abnormally remained in the tissues during ontogenesis, and finally CSCs may be caused by mutations in terminally differentiated cells (Knudson et al., 1973; Sell and Pierce, 1994; Morrison et al., 2002; Jaiswal et al., 2003; Reya et al., 2003; Al-Hajj et al., 2004; Ratajczak, 2005).

Despite advances in detection and treatment of metastatic cancers, applying radiotherapy, chemotherapy, immunotherapy, drug combination, and gene therapy with some vehicles such as viral vectors (Behbod and Rosen, 2005), mortality from cancer remains high (Schultz and Weber, 1999; Stockler et al., 2000; Al-Hajj et al., 2003). The importance of CSCs relies on the potential role of these cells in re-initiation and maintenance of tumor growth, which is the main cause of recurrence and relapse of tumors (Bohl et al., 2011).

It is believed that cancer targeted therapies especially stem cell targeted therapy are superior to current treatments such as traditional chemotherapy or radiotherapy to overcome recurrence, metastasis and chemo-resistance. Commonly used anti-cancer therapies can shrink primary and metastatic tumors, nevertheless such effects are usually transient and relapse of most metastatic cancers frequently occur (Reya et al., 2001), which are attributed

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to CSCs. There is sufficient evidence that CSCs are relatively chemo, radio and endocrine resistant, indicating that novel CSC-targeted therapies are required to achieves a true cure and elimination of cancer (Ablett et al., 2012; Reya et al., 2001). Standard therapies in combination with CSC-targeted therapies could provide effective treatment strategy by de-bulking the tumour mass and preventing recurrence (Chaffer et al., 2011; Gupta et al., 2011).There are several potential ways of targeting CSC including inhibition of self-renewal signaling pathways thus inducing differentiation or apoptosis, targeting resistance mechanisms or targeting the CSC niche that supports them. Monoclonal antibodies raised against specific components of signaling pathways or cell surface antigens on CSCs have been used to target these cells specifically (Ablett et al., 2012). Another important therapeutic option could be development of specific anti-CSC drugs targeting specific markers and pathways (Orian-Rousseau, 2010).

CSCs have been identified and characterized in myeloid leukemia and solid tumors including breast, brain, lung, colon, pancreatic, head and neck cancers (Heppner, 1984; Reya et al., 2001; Al-Hajj et al., 2003; Singh et al., 2003; Dwyer et al., 2007; Li et al., 2007; O'Brien et al., 2007; Prince et al., 2007; Aboody et al., 2008; Nakshatri, 2010), employing typical profile of various surface markers such as CD44, CD133 (Aboody et al., 2008) or ALDH (Balicki, 2007). Breast cancer was the first solid cancer from which CSCs were identified and isolated in combination with flowcytometry by Al-Hajj et al (Al-Hajj et al., 2003; Lindeman and Visvader, 2010). Several studies using the xenograft CSC assay support the CSC model in breast cancer suggesting that ESA+/CD44+/CD24-/low (Al-Hajj et al., 2003; Wicha et al., 2006) or aldehyde dehydrogenase 1 (ALDH1)+ phenotypes may enrich for breast CSCs which this population may be associated with a poorer prognosis (Balicki, 2007; Ginestier and Wicha, 2007; Lindeman and Visvader, 2010; Madjd et al., 2012).

Although considerable progress has been made to identify CSCs, there is still the need to fully characterize the CSCs in terms of cell surface markers. No universal cell surface antigen, or combination of antigens, for the purification of breast CSCs by antibody techniques yet have been identified (Ablett et al., 2012). Many putative CSC markers are not merely restricted to CSCs, therefore the main goal in targeted therapy is specific destruction of CSCs while protecting normal cells (Deonarain et al., 2009).

In addition to surface markers, CSCs share some key signaling pathways with normal stem cells which can be mutated in CSCs and be considered as attractive targets for cancer therapies (Soltanian and Matin, 2011). A distinguished understanding of signaling pathways between normal and CSCs is required to prevent destroying normal stem cells, as this is the key point to perfect accomplishment of anti-CSC therapies (Deonarain et al., 2009).

Signaling pathways including Wnt, Hedgehog (Hh) and Notch play important roles in cell proliferation regulations and contribute to the self-renewal of stem cells and/or progenitor cells in a variety of organs, including the haematopoietic and nervous systems (Austin and Kimble, 1987; Henrique et al., 1997; Chan et al., 1999; Gailani and Bale, 1999; Wechsler-Reya and Scott, 1999; Zhu and Watt, 1999; Polakis, 2000; Zhang and Kalderon, 2001; Al-Hajj et al., 2003).

Mutations of these pathways can contribute to oncogenesis in multiple solid tumors (Reya et al., 2001 Giles et al., 2003; Evangelista et al., 2006; Leong and Karsan, 2006). Wnt pathway hasbeen reported in lung cancer (Liu et al., 2006), colorectal carcinoma (Polakis, 2000) and epidermal tumors (Chan et al., 1999), Hh pathway in medulloblastoma (Wechsler-Reya and Scott, 1999) and basal cell carcinoma (Gailani and Bale, 1999), while Notch pathway mutation has been involved in T-cell leukemia (Ellisen et al., 1991; Reya et al., 2001). Moreover, Hh signaling has been shown to be essential for the self- renewal regulation in normal and human malignant breast stem cells (Liu et al., 2006; Bohl et al., 2011).

One of the novel targeted therapy modalities could be therefore signaling pathways. Blocking an abnormally active Hh pathway using an Hh antagonist in non-small-cell lung cancer (NSCLC) resulted in significant decrease in cell viability and malignancy (Yuan et al., 2007). Also, Notch ligand protein blocking antibody (ADLL4) was used to inhibit the Notch pathway inhuman breast cancer xenografts, leading to a significant reduction of tumor growth and a strong decrease of CD44+breast CSCs (Hoey et al., 2009).

There are two different hypotheses for interaction of stem cells and cancer leading to various applications of stem cells in targeted therapy of breast cancer. The first one is targeting CSC markers or pathways involved in CSCs using monoclonal antibodies as a novel strategy to improve the outcome of cancer therapy (Deonarain et al., 2009). The second one applies stem cells particularly Mesenchymal Stem Cells (MSCs) as promising platform for cell and gene therapy of incurable cancers (Ozawa et al., 2008). The high tropism of MSCs to cancers, as well as their ability to engraft, survive, and proliferate in the tumor without any immunogenicity and toxicity to the host, makes them ideal vehicle for tumor-selective drug delivery. MSCs migrate to sites of tumorigenesis and are utilized as efficient cellular vehicle for the targeted delivery anti-neoplastic therapy to both primary tumors and their metastases (El-Haibi and Karnoub, 2010). Several preclinical studies support the basis for genetically modified MSC to deliver therapeutics to tumor sites; include glioma, melanoma, Kaposi's sarcoma, Ewing sarcoma, as well as carcinomas of the colon, ovary, breast (Studeny et al., 2004; Nakamizo et al., 2005; Khakoo et al., 2006; Komarova et al., 2006; Karnoub et al., 2007; Menon et al., 2007; Coffelt et al., 2009; Duan et al., 2009; El-Haibi and Karnoub, 2010). Despite these approaches, the basic mechanisms involved in the homing of MSCs to sites of malignant growth are still only partially defined.

This systematic review explores the recent burgeoning evidence focusing on these two separate concepts based on selected key words to investigate the application of cancer stem cells as specific targeting modalities in breast cancer and also describes the use of stem cells as cellular

vehicles for breast tumor targeted delivery therapy in the most recent clinical trials.

Materials and Methods

Search strategy

Specific key words were agreed to be searched within Medline (Pubmed), ISI web of knowledge, Gateway, Ovid and Embase for original research articles published between January 2007 and May2012. Included keywords were "breast cancer", "breast neoplasm", "stem cell" combined with "targeted therapy" or "targeted", "therapy" or "therapeutics".

Study inclusion criteria

Published papers were included if the following criteria were met: clinical trials at any phase, either discusses about the application of cancer stem cells as specific targeting modalities in breast cancer or describes the use of stem cells as cellular vehicles for breast tumor targeted delivery therapy, published within the recent five years, English, and predetermined key words existed. Meanwhile, review papers, any type of articles other than original research, exclusive animal experimental research (without additional human experiments), duplicate or sliced research articles, and research protocols were excluded in this systematic review. Review papers, commentaries, editorials, letters, and books were also excluded from the study.

Reference lists of identified papers were reviewed and the Cochrane Libraries was searched for any systematic review in this field or similar subjects. We limited the search to humans, cancer, title/abstract in English papers published within 5 years until May 2012. Abstracts were reviewed by three independent researchers (ZM, EG and EE), then relevant papers were identified and full papers were obtained for scrutiny regarding the methodology and main findings by all authors. Specific parts of the included papers were then entered in a standard table.

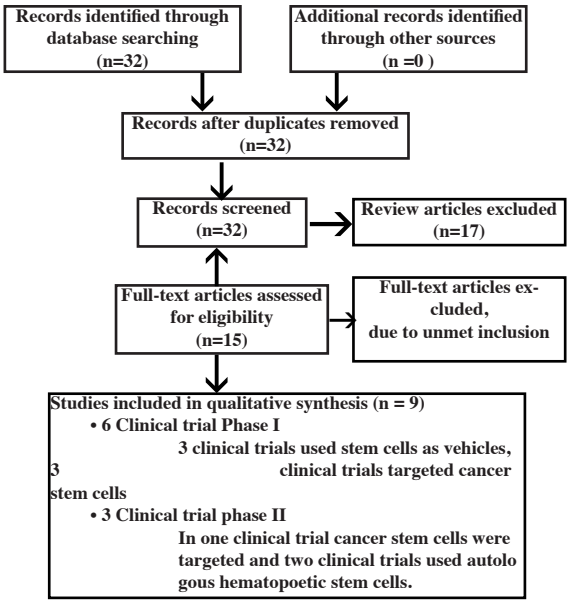

Figure 1. Flow Diagram of the Procedure used to Select Relevant Articles

Search strategy is schemed in Figure 1.

Results

Search results

Nine out of 32 studies, with heterogeneous study design, met the predefined inclusion criteria and all were reviewed Of these, six studies were phase I (Dwyer et al., 2007; Woodward et al., 2007; Vlashi et al., 2009; Grisendi et al., 2010; Dwyer et al., 2011; Milane et al., 2011)and three were phase II clinical trials (Table 1) (Ueno et al., 2009; Resetkova et al., 2010; Viens et al., 2010). There was no published paper regarding phase III clinical trial on stem cell targeted therapy of breast cancer within the period of this study. Although our search investigated all papers over the period of 5 years, the papers possessed our inclusion criteria ranged from Jan 2007 to May 2011. As mentioned earlier, to ease distinguishing both concepts of application of CSCs in breast cancer, the results are presented here separately.

The use of stem cells in combination with therapeutic agents

The role of tumor-secreted monocyte chemotactic protein-1 (MCP-1) in stimulating Mesenchymal Stem Cells (MSC) migration has been studied in a phase I clinical trial by Dwyer et al. (2007) which supports the capability of MSCs as tumor targeted delivery vehicles for therapeutic agents. MSC have been also used as cellular vehicles for tumor targeted delivery of therapeutic agents (Dwyer et al., 2011). Adipose Derived Mesenchymal stromal/stem cells (AD-MSC) armed with TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) has been offered as proficient tools for cell-based gene therapy for incurable cancers (Grisendi et al., 2010).

Autologous stem cell transplantation (HSCT) was applied in combination with 166-Holmium (Ho)-DOTMP to avoid the anticipated myelosuppression confirming that this combination shows an acceptable toxicity profile in bone metastatic breast cancer patients (Ueno et al., 2009).

A randomized clinical trial (RCT) conducted by PEGASE Group evaluated the value of high dose chemotherapy (HDC) in combination with hematopoietic stem cell transplantation (HSCT) and the value of targeted therapies in non-metastatic breast cancer. This study revealed that this combitaion only improve pathological complete response (pCR), while it could not significantly improve overall survival (Viens et al., 2010). The clinical trials in which either stem cell used as gene therapy or HSCT used in combination with chemo/radiotherapy have been summarized in Table 2.

Targeting cancer stem cells

The 26S proteasome as the main regulator of many processes within proliferating cells has been recently introduced for targeting of CSCs. Therefore reduced 26S proteasome activity couldbe applied for identification, tracking, and targeting of this subpopulation (Vlashi et al., 2009).

Wnt/β-catenin signaling pathway in stem/progenitor cells, which is responsible for radioresistency, has been

Table 1. Bibliographic Characteristics of the Included Studies

Author	Journal	Year of publication	Title	Phase of clinical trial
Dwyer et al. (2007)	Clin Cancer Res	2007	Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells	I
Dwyer et al. (2011)	Stem Cells	2011	Mesenchymal stem cell-mediated delivery of the sodium iodide Symporter supports radionuclide imaging and treatment of breast Cancer	I
Grisendi et al. (2010)	Cancer Res	2010	Adipose-derived mesenchymal stem cells as stable source of tumor necrosis factor–related apoptosis-inducing ligand delivery for cancer therapy	I
Vlashi et al. (2009)	JNCI	2009	In vivo imaging, tracking, and targeting of cancer stem cells	I
Woodward et al. (2007)	PNAS	2007	WNT/β-catenin mediates radiation resistance of mouse mammary progenitor cells	I
Milane et al. (2011)	PLoS ONE	2011	Therapeutic efficacy and safety of paclitaxel/lonidamine loaded EGFR-Targeted nanoparticles for the treatment of multi-drug resistant cancer	I
Ueno et al (2009)	Clinical Breast Cancer	2009	Pilot study of targeted skeletal radiation therapy for bone-only metastatic breast cancer	II
Resetkova et al. (2010)	Breast Cancer Res Treat	2010	Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment	II
Viens et al. (2010)	Cancer	2010	Systemic therapy of Inflammatory breast cancer from high-dose chemotherapy to targeted therapies	II

introduced as an attractive target for directed anti-stem cell therapy by Woodward et al. (2007).

Multi-drug resistant (MDR) cancer, known as cancer cell with stem cell properties, was targeted with a nanocarrier system by binding to the EGFR receptor and subsequently delivered drug solutions, paclitaxel (PTX) (Milane et al., 2011) and lonidamine (LON) to the site of a tumor. These EGFR targeted combination nanoparticles decreased tumor volume and also expression of hypoxic and MDR associated proteins in the orthotopic breast cancer model. This nanocarrier system could be used as a model for the design of other MDR cancer therapies (Milane et al., 2011).

Another study conducted by Resetkova et al to investigate the relevance of ALDH1 as a putative cancer stem cell marker in breast cancer, did not demonstrate any correlation between ALDH1 expression with response to neoadjuvant therapy or overall survival after chemotherapy, in breast cancer patients (Resetkova et al., 2010).The clinical trials (phase I and II) in which CSCs were targeted with different mechanisms, have been summarized in Table 3.

Discussion

There are two major concepts regarding interaction of stem cells and cancer. This discrepancy was the main cause of heterogeneity in study designs that we were encountered in this systematic review. In the present study, we identified nine recent clinical trials based on predetermined criteria, in which either stem cells applied in combination with therapeutic agents as cellular vehicles or CSCs targeted for breast cancer therapy. Therefore we discuss different concepts of "cancer" and "stem cells" under various sub headings:

The use of stem cells in combination with therapeutic agents, in the first series of studies, MSCs were applied as vehicle to transfer therapeutic agent in incurable breast cancers. Dwyer et al. (2007) investigated the role of tumor-secreted MCP-1 in stimulating MSC migration in a phase I clinical trial, indicating the important role of MSCs as a vehicle for in vivo tumor-targeted therapy because of specific migration to tumors. They determined systemic levels of the chemokine in a cohort of breast cancer patients and age-matched controls concluding that MCP-1levels were significantly higher in postmenopausal breast cancer patients than the age-matched control group, supporting the capability of MSCs as tumor-targeted delivery vehicles for therapeutic agents. Although this data supports a potential role for MSCs as attractive delivery agents in tumor-targeted therapy, further studies are needed to clarify the factors that facilitate MSC migration and engraftment to provide the clinical application of this novel approach.

Moreover, in a recent study Dwyer et al. (2011) showed that MSC-mediated expression of the sodium iodide symporter (NIS) is potentially is appropriate for imaging, tracking and therapy of breast cancer. By injection of MSC-NIS, human NIS (hNIS) gene expression in various tumor sites occurred and a significant reduction of tumor growth was observed. The major advantage of this strategy was the ability to track MSCs migration noninvasively before therapy, supporting the application of MSCs as a vehicle in novel therapy of breast cancer. The persistence of MSCs after treatment and their role in the tumor microenvironment is still unclear. Further improvement

Table 2. The use of Stem Cells as Delivery Vehicle/Autologous Hematopoetic Stem Cells in Combination Therapy

Authors	Methods	Findings	Comments
Dwyer et al. (2007)	• Injection of fluorescently labeled MSCs to mice with breast cancer (BC) • Detection of MSC migration in response to primary tumors in vivo and in vitro. • Measuring of monocytechemotactic protein-1 (MCP1) in serum samples of 125 breast cancer patients and 86 healthy controls.	• Significant increase in MSC migration was seen in response to primary BC in vitro. • Significant reduction in MSC migration to tumors caused by using MCP-1 antibody. • Significantly higher levels of serum MCP-1was found in post menopausal BC patients compared to controls.	**Strength**: • This study indicates a role for tumor-secreted MCP-1in stimulating MSC migration, which supports the potential of MSCs as attractive delivery vehicles in tumor-targeted therapy. **Weakness**: • Although MSCs localized around the border of the tumor, these cells were morphologically intact and survived up to 72 h after administration, which warrants further studies to evaluate the differentiation status of engrafted MSCs.
Dwyer et al. (2011)	• Contamination of isolated MSCs from bone marrow using adenoviral vector and detection of NIS expression using relative quantitative-PCR (RQ-PCR). • injection of MSCs-NIS in tumor bearing mice. • Imaging of animal tumoral tissue after injection of MSC-NIS. • Studying of in vivo biodistribution of NIS-expressing MSCs by RQ-PCR in harvested mice organs. • Evaluation of tumor size. • HandE and IHC staining of tissue sections to study of necrotic areas in tumors harvested from mice after therapy	• Uptake of tracer was noticeable at the site of the tumor by day 14 • hNIS gene expression was observed in the intestines, heart, lungs, and tumors at early days but later depleted in non-target tissues and persisted at the tumor site. • Significant reduction in tumor size was observed in mice after injections of MSC-NIS followed by saline or 131I therapy. • Necrotic areas in tumors was observed in mice treated with 131I 14 days after MSC-NIS delivery in HandE staining	**Strength**: • The major advantage of this strategy is the ability to track MSCs migration noninvasively before therapy. • persistent functional NIS expression suggests that MSCs are not proliferated at the tumor site. • Implying MSCs as a vehicle in novel therapy of breast cancer was supported by this study. **Weakness**: • The persistence of MSCs after treatment and their role in the tumor microenvironment is unclear. Further improvement could be achieved by repeated dose of MSC-NIS and radioiodide, because irradiation causes stimulation for MSC engraftment.
Grisendi et al. (2010)	• HeLa cells were cultivated in DMEM. Primary tumor samples obtained from lung cancer patients. Trypsinized cells were spun onto slide and stained by HandE method. • Isolation and transduction of human AD-MSCs were conducted with a bicistronic murine stem cell virus derived retroviral vector (pMIGR1) encoding green fluorescent protein (GFP) and TRAIL. • Fluorescence-activated cell sorting analysis (FACS) was carried out by staining of the cells with PE-anti-TRAIL, PE-anti-TRAIL-R1/DR4, PE- anti-TRAIL-R2/DR5 and isotype controls. • Intracellular staining on transduced AD-MSC and controls were performed with BD Cytofix/Cytoperm kit using the PE-anti-TRAIL antibody. • TRAIL was measured using Quantikine Human TRAIL/ TNFSF10 kit by ELISA method. • The apoptotic activity of caspase-8 was assessed by FACS with the CaspGLOW Red Active Caspase-8 Staining kit. • In vivo study was performed by flank injection of AD-MSC TRAIL and AD-MSC GFP in 6 groups of mice. • GFP-marked AD-MSCs were monitored in excised and processed tumors by PCR. • Histology staining (HandE and IHC) was performed.	• FACS analyses demonstrated that wild Type AD-MSC (WT-MSC) and AD- MSC GFP do not constitutively express TRAIL; but, gene modification of AD-MSC (GM-MSC) with TRAIL-encoding vector revealed a relevant protein expression by surface and intracellular staining. • In PI staining by FACS, there were no differences in TRAIL toxicity on cell death between confluent WT AD-MSC, AD-MSC GFP, and AD-MSC TRAIL. • ELISA measuring TRAIL released in culture by confluent AD-MSC GFP versus AD-MSC TRAIL at different time points. • AD-MSCs expressing TRAIL displayed anti-tumor activity in Hela cell lines. • AD-MSC TRAIL prevented tumor growth inxeno-trasplanted mice compared with another cells without TRAIL. • AD-MSC expressing TRAIL induced apoptosis in primary tumor cells by cell-to cell contact via caspase-8 activation. • Localization of AD-MSC TRAIL into tumor site was observed by IHC.	**Strength**: • This study introduces a novel cancer gene therapy based on AD-MSC directly producing a potent proapoptotic agent (TRAIL). • TRAIL mediates the apoptotic effect by binding to its death receptors (DR), causes caspase-8 activation, triggering apoptosis. • The presence of DR on AD-MSC, could affect cell survival after TRAIL autocrine production, therefore, human AD-MSC could be an ideal vehicle to deliver TRAIL. • Treatment with AD-MSC TRAIL was more effective than recombinant TRAIL (rTRAIL), suggestingthat AD-MSC acts as a powerful tool in cancer therapy to inhibit cancer growth. • Bortezomib could be combined with a cell-based TRAIL delivery to successfully target TRAIL-resistant cancers. • A cell therapy with AD-MSC TRAIL alone or in combination with sensitizing agents (Bortezomib) could be a novel therapy for incurable cancers. **Weakness**: • AD-MSCs without gene modification could not constitutively produce TRAIL
Ueno et al. (2009)	• Six women aged <65yearswith bone-only metastatic breast cancer • A high-dose radio pharmaceutical agent (166 Holmium (Ho)-DOTMP) used in combination with HSCT target bone metastases in breast cancer. • The activity of 166Ho-DOTMP was measured to deliver a therapeutic absorbed dose of 22 Gy (n = 3) or 28 Gy (n = 3) to bone marrow. • Treatment was followed by HSCT to avoid the myelosuppression. • Median follow-up time was 40 months.	• All patients have prompt hematologic recovery. • None of patients experienced acute toxicity of166Holmiumexcept myelosuppression. • Low acute toxicity profile and complete response was observed in 2 of 6 patients. • Two patients showed progression free without evidence of disease for more than 6 years. • Five cases experienced disease relapse (4 at extra osseous sites) and died of progressive disease. • Median time to progression was 10.4 months.	**Strength**: • Low acute toxicity profile and good progression-free survival may indicate achieving long-term remission. • Lack of GVHD in the autologous setting and the use of a targeted bone therapy **Weakness**: • As 166Ho-DOTMP is no longer available, the commercially available agent, 153Sm-EDTMP, is currently used in clinical trials which may influence the results. • The small number of patients limits the evaluation of incidence of other adverse effects or the likelihood of developing secondary hematologic malignancies.
Viens et al. (2010)	• 380 patients with non-metastatic IBC have been included in a series of multicentric clinical trials, where the value of using HDC with HSCT was examined. • 3 out of 5 trials registered 329 patients, who were under HDC with HSCT. • PEGASE-2: included 100 patients, who received 4 cycles of chemotherapy, 87 women completed treatment, revealed decreased rates of pCR and 3-year survival. • PEGASE-5, on 54 patients, received 7 cycles of HDC with HSCT. Study was prematurely stopped because of toxicity, infection and 2 deaths. • PEGASE-7: on 175 patients. Trial was closed on June 2005. • Two remaining trials combine targeted therapies with conventional dose chemotherapy in ERBB2-negative (Beverly 1 trial; bevacizumab) and ERBB2-positive (Beverly 2; bevacizumab and trastuzumab) IBC.	• PEGASE 02 and PEGASE 05 showed a high pathological complete response (PCR) rate after primary regular HDC, recommending that there is no benefit in applying more than 4 cycles of HDC. • PEGASE 07 tested adjuvant maintenance chemotherapy after neo-adjuvant HDC.	**Strength**: • HDC combined with HSCT could be an experimental approach leading to high pCR rates and may have benefits to subgroups of patients. • Targeted therapies, such as anti-ERBB2 (trastuzumab) and anti-angiogenic (bevacizumab) drugs may improve survival in non-IBC. **Weakness**: Huge heterogeneity of IBC at the biological level with the existence of molecular subtypes similar to those described in non-IBC. • The heterogeneity of this rare but aggressive disease should be accounted in the design of future clinical and translational studies.

Table 3. Targeting Cancer Stem Cells

Authors	Methods	Findings	Comments
Vlashi et al. (2009)	• Human glioma and breast cancer cell lines were engineered to express ZsGreen fused to a carboxy terminal degron of ornithine decarboxylase (ZsGreen-cODC and TK-ZsGreen-cODC fusion proteins) using retroviral transduction that accumulates in cells in the absence of 26S proteasome activity. • Proteasome function and proteolytic activities of chymotryptic, tryptic, and caspase enzymes were evaluated and monitored by measuring the release of the fluorescent group, 7-amido-4-methylcoumarin (AMC) using a fluorescence plate reader. • Proteasome subunit expression in cells expressing the fusion protein was evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR). • The stem cell phenotype of CICs was determined by a sphere formation assay, by immunohistochemical staining for known stem cell markers in vitro, and by in vivo analyzing their tumorigenicity in nude mice. • CICs were tracked by in vivo fluorescence imaging for the macroscopic presence of ZsGreen-positive cells in the tumors using the Maestro In-Vivo Imaging System after radiotherapy of tumor bearing mice and targeted specifically through a thymidine kinase degron fusion construct.	• CSCs had decreased proteasome activity relative to the respective monolayers. • Low proteasome activity cancer cells can be monitored in vitro and in vivo by the accumulation of ZsGreen-cODC that targets it for 26S proteasome degradation. • In vitro, ZsGreen-positive cells had increased sphere-forming capacity, expressed CSC markers, and lacked differentiation markers compared with ZsGreen-negative cells. • In vivo, ZsGreen-positive cells were approximately 100-fold more tumorigenic than ZsGreen-negative cells when injected into nude mice. • The number of CICs in tumors increased after 72 hours post radiation treatment. • CICs were selectively targeted via a proteasome-dependent suicide gene (Using a TK-ZsGreen-cODC vector) leading to tumor regression.	**Strength**: • This study demonstrates how to identify and track CICs in animal models of cancer which allows improved assessment of therapeutic approaches compared to conventional methods like measuring tumor response. **Weakness**: • CSC population with low protease activity may be a heterogenous population that needs further identification. • This study may underestimate the difference in tumorogenicity between ZsGreen-positive and ZsGreen-negative cells. • Long term experiments may be required to detect the possible transformation ofZsGreen-negative to positive cells. • To obtain a pure CSC population ZsGreen-positive cells need to be further purified.
Woodward et al. (2007)	• Mammary epithelial cells (MECs) and breast cancer cell line (MCF-7) were isolated from BALB/c mice, cultured, irradiated, and analyzed for Side population (SP) by Hoechst 33342 staining and flow cytometry for Sca1 expression, also for CD24+ CD29+ population by flowcytometry. • Clonogenic assays were performed to investigate the effect of radiation in cell killing. Cell cycle assay was carried out after radiation treatment to confirm that the Sca1+ cells are cycling. Sorted Sca1+and Sca1-population stained with 7-amino-actinomycin D and pyronin Y to distinguish between G0 and G1. • Sca1+ and Sca1- cells from MECs were sorted after irradiation and immunostained with anti-phospho-H2AX antibody to examine DNA breaks. • To determine the role of stem cell survival factor Wnt/β-catenin in radioresistancy of SP cells, MECs from Wnt-1 transgenic mice and wild-type mice of the same background cultured, irradiated and stained with Hoechst 33342 for %SP analysis by using flow cytometry. • Real-time PCR for survivin expression was carried out after irradiation in Sca1+ and Sca1-cells. • Sca1+ cells stained with anti-nonphospho-β-catenin phycoerythrin (PE) that binds to activated β-catenin by using flowcytometry.	• Radiation selectively increased the progenitor fraction (%SP) in both MECs and MCF-7 cells, also increased Sca1+ (progenitor) fraction within the SP by killing the more sensitive Sca1-(non progenitor) cells. • Radiation increased percentage of CD24+ CD29+ cells from MCF-7 cells but not uncultured MECs. However, radiation selectively decreased the lin CD24+ CD29low fraction cells. The CD24+ CD29+ population was sensitive to radiation. • Radiation induced more DNA damage in Sca1- cells after irradiation. • Radiation selectively activated β-catenin and survivin in Sca1+ cells. • Survivin expression was selectively increased in Sca1+ cells in response to radiation. • β-catenin is selectively activated in Sca1+ cells comparison with negative Scal cells. • β-catenin over expression may enhance cell survival after radiation treatment through regulating surviving	**Strength**: • This study emphasized that progenitor cells in the mammary glands are more resistant to clinically relevant doses of radiation than non-progenitor cells, and that over-expression of the Wnt/β-catenin pathway can enhance the radio-resistance of progenitor cells. • Wnt/β-catenin signaling pathway, as an attractive target for directed anti-stem cell therapeutics. • The higher doses of radiation can significantly decline progenitor cells suggesting that 6 Gy is sufficient to kill both progenitor and non-progenitor cells. • Freshly isolated primary MECs were used to avoid confounding the results **Weakness**: • β-catenin is not commonly mutated in human breast cancers.
3.Milane et al. (2011)	• Nude mice with MDR breast tumor were treated with EGFR-targeted, polymer blend nanoparticles loaded with paclitaxel and lonidamine. • This nanoparticle formulation is internalized via the EGFR receptor; treatment results in a cascade of cellular changes and a reduction of tumor size. • The safety/ toxicity of this treatment were evaluated by measuring the change in tumor size, body weight, plasma levels of the liver enzymes, WBC and platelet counts. • Hypoxic and MDR markers (EGFR,; HIF, hypoxia inducible factor; HXK2, hexokinase 2; Pgp, P-glycoprotein; SCF, stem cell factor) were measured using IHC method.	• Treatment with EGFR-targeted LON/PTX NPs was more effective than combination SOL treatment. • Toxicity of SOL treatment was much higher compared to NP treatment. • The combination NP s resulted in less reduction in body weight and more recovery in body weight, less LDH, less ALT, lower WBC counts, and higher platelet counts. • LON/PTX therapy with NPs resulted in less liver toxicity. • The expression of MDR markers after treatment with combination NPs was decreased.	**Strength**: This nano-carrier system actively targets MDR cells by binding to the EGFR receptors and subsequently delivers PTX and LON to tumor site. • Treatment with EGFR-targeted combination nano-particles decreased tumor density, altered the MDR phenotype of the tumor xenografts and were considerably less toxic than solution treatments. • The nano-carrier system could be used for the development of other MDR cancer therapies; due to the flexibility and simplicity of design, this system is an advanced tailored medicine. **Weakness**: More in vivo experiments are needed to approve this combination.
Resetkova et al. (2010)	• The prognostic relevance of cancer stem cell marker, ALDH1, was assessed in four cohort groups including 245 adjuvantly treated invasive breast cancers, 34 neoadjuvantly treated and two groups of 58 and 40 triple negative cases using immunohistochemistry. • Both tumor cell and stromal expression of ALDH1 were evaluated.	• ALDH1 expression was significantly correlated with tumor grade in the neoadjuvant cohort. • There was no significant enhancement for ALDH1 positive cells in the post-neoadjuvant therapy specimens compared to pretreatment samples. • The higher level of ALDH1 stromal expression was significantly correlated with best disease-free survival as well as a trend for overall survival.	**Strength**: • The association of higher stromal expression of ALDH1 with disease-free survival suggests that tumor microenvironment may play a significant role in determining the prognostic impact of stem/progenitor cells in human breast tumors. **Weakness**: There was no correlation between ALDH1in breast tumor cells with response to neo-adjuvant therapy survival, following adjuvant chemotherapy. Therefore ALDH1 not recommended as a useful marker for targeted therapy of breast cancer.

might be achieved using repeated dose of MSC-NIS and radioiodide, as other studies have demonstrated that tumor irradiation stimulates increased MSC engraftment (Klopp et al., 2007; Spaeth et al., 2008; Zielske et al., 2009; Kim et al., 2010). This approach may cause greater stimulation of MSC engraftment in remaining disease and could be applied as an effective treatment in metastatic cancer.

In another study AD-MSC has been used as cellular vectors to deliver proapoptotic molecules TRAIL for breast cancer treatment (Grisendi et al., 2010). Although antitumor activity of recombinant human TRAIL has been confirmed in several studies (Grisendi et al., 2010), its application in vivo is limited by a short half- time in plasma, due to a rapid renal clearance. To overcome this limitation, stably transduced AD- MSC used as a constant source of TRAIL production targeting a variety of tumor cell lines including breast cancer. AD-MSC TRAIL is localized into tumors and mediated apoptosis without extensive toxicities to normal tissues after injection into mice. In spite of liver toxicity of recombinant TRAIL (Jo et al., 2000), the functional liver enzyme were normal in TRAIL- treated mice. This study indicated that cell therapy with AD-MSC TRAIL alone or in combination with sensitizing agents (Bortezomib) successfully targets TRAIL-resistant cancers, which is a new potential strategy in cancer therapy (Grisendi et al., 2010).

These studies confirm the notable experimental possibilities of MSC-based antineoplastic cellular therapy, and underline their potential application in breast cancer treatment. Some of these approaches are already in various phases of clinical trials; however, their efficacy and clinical safety in breast cancer patients remain to be determined.

Based on our search strategy we found a series of clinical trial phase II in which stem cells applied in combination with radiotherapy or chemotherapy to enhance their efficacy. For example Ueno et al determined the safety and efficacy of radiopharmaceutical agent, named 166-Holmium (Ho)-DOTMP, for irradiating malignant cells and adjacent marrow in bone metastatic breast cancer women (Ueno et al., 2009). This finding confirms that 166-Ho-DOTMP in combination with autologous stem cell transplantation had an acceptable toxicity profile when used in bone-metastatic breast cancer. Two out of 6 patients remained progression free without evidence of disease for more than 6 years, achieving long term remission (Ueno et al., 2009). Evaluation of other side effects and the probability of secondary hematologic malignancies were limited in this study due to small number of cases.

In a more recent study, a collection of RCTs by PEGASE Group (Viens et al., 2010) have been conducted in France to examine the value of high dose chemotherapy (HDC) with hematopoetic stem cell transplantation (HSCT) and the vlaue of targeted therapies in non metastatic inflammatory breast cancer (IBC), which revealed an appropriate pathological complete response rate by HDC. The other parts of these ongoing clinical trials recently have been published which was not in the time frame of this study (Viens et al., 2010).

HDC combined with autologous HSCT has been applied in several solid tumors to overcome tumor chemoresistance, indicating that this combination may improve tumor response rates or relapse-free survival (RFS), especially in selected subsets of patients (Banna et al., 2007). However, Banna et al. (2007) reviewed solid tumor trials concluding that there was no overall benefit for the use of this combination.

In a more recent systematic review Berry et al concluded that combined use of HDC with HSCT prolonged RFS and overall survival (OS)in high-risk primary breast cancer compared with control, whereas a statistically significant benefit was not observed in OS (Berry et al., 2011).

Targeting cancer stem cells, cancer stem cells are of particular interest in the literature, for their ability in initiation and maintenance of tumor growth and their potential role in early relapses and resistance to current therapies (Reya et al., 2001; Heppner, 1984; Al-Hajj et al., 2003; Clarke et al., 2006; Massard et al., 2006; Dwyer et al., 2007; Aboody et al., 2008; Nakshatri, 2010; Bohl et al., 2011; Lehmann et al., 2011). Despite nearly a decade after the introduction of tumorogenicity of CSCs in breast cancer (Al-Hajj et al., 2003), only a few clinical trials have been performed to confirm this hypothesis. In the second group of studies, cancer stem cells were targeted via their characteristics such as markers or signaling pathways. In a study by Vlashi et al. (2009) the 26S proteasome as the main regulator of many processes within a proliferating cell has been applied for imaging, tracking and targeting of CSCs. Reduced proteasome activity may occur simultaneously with the expression of stem cell markers and lack of differentiation markers (Vlashi et al., 2009). CSCs may be either immunologically silent or express antigens leading to failure in current targeted immunotherapy approaches. This system enables screening of novel compounds thatmight alter 26S proteasome function specifically in CSCs leding to novel targeted therapies against this subpopulation. Therefore reduced 26S proteasome activity could be assumed as a general feature of CSCs and could be easily used to identify, track and target this subpopulation in vitro and in vivo (Vlashi et al., 2009). Although CSCs can be selectively targeted via a proteasome- based dependent suicide gene, this population with low protease activity may be a heterogenous population that needs further identification. To obtain a pure CSC population, ZsGreen-positive cells are required to be further purified.

The Wnt, Notch and Hedgehog (Hh) pathways are developmental pathways that are commonly activated in different cancer types. The mutation of these pathways have been frequently occurred in many types of cancers particularly within subpopulation of CSCs (Dickinson and McMahon, 1992; Kintner, 1992; Ruiz i Altaba, 1999; Weissman, 2000; Reya et al., 2001; Barker and Clevers, 2006; Visvader and Lindeman, 2008; Shackleton et al., 2009; Curtin and Lorenzi, 2010; Li and Clevers, 2010; Snippert et al., 2010). This finding provides an opportunity for specifically targeting CSCs which are responsible for tumor initiation, progression, recurrence and metastasis (Curtin and Lorenzi, 2010). Significant progress has been made in developing therapeutics targeting Notch and Hh

(Luistro et al., 2009; Robarge et al., 2009), whereas the Wnt pathway has been more challenging for targeted therapy (Curtin and Lorenzi, 2010).

Wnt/β-catenin signaling pathway has been suggested to be an attractive target for directed anti-stem cell therapy (Woodward et al., 2007). The subpopulation of stem/progenitor cells which remain after breast cancer radiotherapy, may lead to recurrent disease. It was hypothesized that radio resistance of this subpopulation is partially mediated by Wnt/β-catenin signaling pathway, which is involved in stem cell survival. Thus, radioresistance of CSCs was investigated by treating primary BALB/c mouse mammary epithelial cells with clinically relevant doses of radiation which resulted in enrichment of normal progenitor cells (Woodward et al., 2007). This data confirm that progenitor cells in the mammary gland are more resistant to radiation compared to non-progenitor cells, indicating that overexpression of the Wnt/β-catenin pathway may enhance the radioresistance of stem/progenitor cells. Therefore, targeting Wnt/β-catenin pathway that is responsible for self-renewal can be a potential therapeutics strategy (Woodward et al., 2007). Although mutation of β-catenin may not commonly occurred in human breast cancers, various studies showed the role of Wnt signaling pathway in pathogenesis of breast cancer (Lin et al., 2000; Jain et al., 2002; Wong et al., 2002; Klopocki et al., 2004) suggesting a link between Wnt signaling and DNA damage response in epithelial cells (Ayyanan et al., 2006).

As a result of its role in different cancers, the Wnt signaling pathway is a major target for therapeutic intervention. Wnt inhibition could be used in combination with classic chemotherapeutic agents ;i.e if the CSCs were targeted accompanied with a Wnt pathway inhibitor, more effective response would be expected (Curtin and Lorenzi, 2010).

Some triple negative breast cancer cell lines have been shown to express ligands and markers of abnormal Wnt/β-catenin signaling without common mutations in the pathway. Wnt signaling in these cells can be inhibited by overexpression of endogenous inhibitors (Bafico et al., 2004). Moreover, the Wnt pathway regulates epithelial-mesenchymal transition (EMT), an important component of metastasis. Therefore it can be hypothesized that the Wnt pathway in breast CSCs may offer an exceptional opportunity to target metastasis as a main cause of morbidity in many types of cancers (Kim et al., 2002; Muller et al., 2002; Liebner et al., 2004; Thiery et al., 2009; Curtin and Lorenzi, 2010). Another clinical challenge in conventional therapy of cancer is the development of multi-drug resistant (MDR) cancer that often slow down the treatment as it results in metastatic disease (Harris and Hochhauser, 1992; Yague et al., 2007). Because MDR is resistant to many current therapies, there is a demand for new drug combinations with less toxicity for treating MDR.

Two models of cancer stem cells have been proposed:cancer initiating stem cells, which originate as stem cells, but alter into cancer causing cells; and cancer derived stem cells, which are a population of cancer cells with stem-like properties, which known as MDR cells (Milane et al., 2011). In line with the second model, various studies have shown that cell stressors such as hypoxia, which are capable in inducing cancer aggression and MDR phenotypes, also increase stem-like properties (Harris, 2002; Semenza, 2003; Cosse and Michiels, 2008; Han et al., 2008; Semenza, 2008). In addition, many MDR cells over-express epidermal growth factor receptor (EGFR) (Franovic et al., 2007). Milane et al utilized this expression through development of EGFR-targeted, polymer blend nano-carriers to combat MDR cancer using paclitaxel (PTX) (Milane et al., 2011) and lonidamine (LON) (Del Bufalo et al., 1996; Ravagnan et al., 1999; Li et al., 2002),where a novel orthotopic model of MDR human breast cancer in nude mice was developed to evaluate the safety and efficacy of nano-particle (NP) treatment. They observed that treatment with the EGFR-targeted LON/PTX nano-particles decreased tumor density and altered the MDR phenotype of the tumor xenografts, which means that combination of LON/PTX therapy using EGFR-targeted NPs could be used as a new approach for the treatment of MDR cancer. Although this approach provides a solution to chemotherapy related toxicity through the use of a nano-carrier system, more in vivo experiments are needed to approve this combination (Milane et al., 2011).

To evaluate the prognostic impact and relevance of ALDH1 as a putative cancer stem cell marker in breast cancer (Resetkova et al., 2010) four cohorts series including an adjuvantly treated series of 245 invasive cancers, a neoadjuvantly treated series of 34 cases, and two series of 58 and 40 triple negative cases were studied by immunohistochemistry (IHC). In spite of prevoius studies that stated the role of CSCs in resistance to chemotherapies, this study showed that unexpected expression of ALDH1 in breast tumor cell did not correlate with response to neoadjuvant therapy, disease-free or overall survival following adjuvant or neoadjuvant chemotherapy. Therefore,based on Resetkova's study ALDH1 can not be suggested as a useful marker for targeted therapy of breast cancer (Resetkova et al., 2010).

Currently, two methods used to measure breast CSC activity include FACS using antibodies to cell surface markers or intracellular enzymes such as ALDH, using the ALDEFLUOR assay, and mammosphere assay. A previous immunohistochemical (IHC) study performed by Ginestier et al in a large series of breast cancer patients, demonstrated a correlation between ALDH1 expression and poor prognosis (Ginestier et al., 2007).Whether this is because of increased number or activity of breast CSCs remains unknown. Therefore, prior to application of IHC markers as alternative measures of breast CSC expression in clinical trials, they first need to be correlated with functional assays of CSC activity (Ablett et al., 2012). The clinical effectiveness of putative breast CSC markers such as ALDH1, CD44 and CD24 to identify and monitor breast CSCs using IHC is complicated due to heterogeneous nature of disease and the existence of different populations of breast CSCs within a single tumour (Park et al., 2010).

In contrast, one may critique that the present method for evaluating CSCs by injecting cells into mice may introduce a selection bias for human cells capable of

surviving and proliferating in the mouse microenvironment with foreign growth factors and cytokines (Kelly et al., 2007; Quintana et al., 2008; Ablett et al., 2012).

Recently the merged model of the clonal evolution model and the CSC hypothesis has been proposed in order to clarify tumour maintenance and progression, which predicts that the frequency of CSCs varies significantly in each patient and appears to be dependent on the type of breast cancer and the dominant mutations, gene amplifications and deletions (Ablett et al., 2012).

In order to assess breast CSCs in clinical trials, it is important to use reliable methods for identification and isolation of breast CSCs, as well as developing novel therapeutic endpoints which reflect their expression and/or function. Therefore, novel clinical settings should be able to measure CSC frequency as well as tumour volume. Moreover, a clinical test must have a high sensitivity and specificity, be acceptable to the patient, logistically reasonable, quick to perform and cost-effective (Ablett et al., 2012).

Tumour formation and serial transplantation assays which have been used the 'gold standard' in vivo methods for measuring breast CSC activity, are technically challenging, expensive, possessing ethical consequences, and would be impractical to apply in clinical settings. However, alternative in vitro methods such as colony-forming assays and identification of cell surface markers have been employed in pre-surgical window trials to assess the efficacy of treatments on the breast CSCs before and after treatment (Yu et al., 2007; Li et al., 2008). Though, the technical expertise, time and expense for implementing these assays limits their application in large scale clinical settings (Ablett et al., 2012).

Study publication bias, publication bias, as a matter of limitation in any systematic review, should be considered in our study, where research with positive and desirable results is potentially more attractive to be published. Considering predetermined inclusion and exclusion criteria, likewise other systematic reviews, restricted the occurrence of selection bias.

In conclusion, this systematic reviewinvestigated two different models concerning stem cells and cancer based on specific key words demonstrating an ever increasing experimental data on various applications of stem cells in targeted therapy of breast cancer.

In the absence of large prospective clinical trials so farto investigate mid and long term outcomes, a few conclusions canbe drawn from this systematic review. Applying MSCs as potenial vehicles to transfer therapeutic agents could be a contemporary therapeutic approach for breast cancer. However, this approach remains an experimental treatment and needs to be confirmed in large scale randomized clinical trials. In addition, combination of hematopoietic stem cell transplantation (HSCT) with high-dose chemotherapy (HDCT) in breast cancer patients could not significantly improve overall survival.

Novel CSC-targeted therapies in breast cancer are very challenging due to heterogenecity of disease. The major challenge is to determine which of CSC properties could be targeted or which CSC biomarkers are appropriate to measure the efficacy of the novel CSC therapies. Although utilizing biomarkers had been proposed to identify and assess CSC activity in clinical trials, there are no universal molecular marker to isolate breast CSCs properly and suitable for use in clinical trials, but they will be achievable in near future. It could be concluded that there are clear uncertainties over the clinical application of stem cells in targeted therapy of breast cancer, yet warrants confirmation in appropriately designed controlled trials.

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