Molecular Biomarkers of Cancer Stem/Progenitor Cells Associated with Progression, Metastases and Treatment Resistance of Aggressive Cancers

Murielle Mimeault and Surinder K. Batra

Department of Biochemistry and Molecular Biology, Fred & Pamela Buffet Cancer Center, Eppley Cancer Institute, University of Nebraska Medical Center, Omaha, NE 68198-5870, U.S.A.

Corresponding authors: Murielle Mimeault, Ph.D., and Surinder K. Batra, Ph.D.

Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870, U.S.A. Phone: (402)559-5455; Fax: (402)559-6650; Email: mmimeault@unmc.edu and sbatra@unmc.edu

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Running title: Molecular gene signatures of aggressive cancers

Keywords: Cancer, Cancer stem/progenitor cells, Cancer-initiating cells, Metastases, Metastasis-initiating cells, Molecular biomarkers, Gene signature, Exosomes, Circulating tumor cells, Disseminated tumor cells, Therapeutic targets, Personalized medicine.

Abbreviations: ABC, ATP-binding cassette; ALDH, aldehyde dehydrogenase; BCSCs, breast cancer stem cells; BTSCs, brain tumor stem-like cells; CRPCs, castration resistant prostate cancers; CTCs, circulating tumor cells; CXCR4, CXC chemokine receptor 4; DFF, dean flow fractionation, EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; ER, estrogen receptor; FASN, fatty acid synthase; FDG-PET, 2[18F]fluoro-2-deoxy-D-glucose-positron emission tomographic; GBM, glioblastoma multiforme HIFs, hypoxia-inducible factors; HSP, heat-shock protein; MET, hepatocyte growth factor receptor; MACS, magnetic activated cell sorting; MAPK, mitogen-activated protein kinase; MIC-1, macrophage inhibitory cytokine-1; miRNA, microRNA; mTOR, molecular target of rabamycin; MUC1, mucin 1; NF-κB, nuclear factor-kappaB; PC, prostate cancer; PDAC, pancreatic ductal adenorcinoma; PI3K, phosphatidylinositol-3' kinase; PSA, prostate-specific antigen; PTCH1, patched receptor 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; SCID, severe combined immunodeficient; SHH, sonic hedgehog; SP, side population; VEGF, vascular endothelial growth factor and VEGFR2,vascular endothelial growth factor receptor 2.

Abstract

The validation of novel diagnostic, prognostic and predictive biomarkers and therapeutic targets in tumor cells is of critical importance for optimizing the choice and efficacy of personalized therapies. Importantly, recent advances have led to the identification of gene-expression signatures in cancer cells, including cancer stem/progenitor cells, in the primary tumors, exosomes, circulating tumor cells (CTCs) and disseminated cancer cells at distant metastatic sites. The gene-expression signatures may help to improve the accuracy of diagnosis and predict the therapeutic responses and overall survival of cancer patients. Potential biomarkers in cancer cells include stem cell-like markers (CD133, aldehyde dehydrogenase "ALDH", CD44 and CD24), growth factors and their cognate receptors (epidermal growth factor receptor "EGFR", EGFRvIII and HER2), molecules associated with epithelial-mesenchymal transition "EMT" (vimentin, N-cadherin, snail, twist and Zeb1), regulators of altered metabolism (PI3K/Akt/molecular target of rabamycin "mTOR") and drug resistance (multidrug transporters and macrophage inhibitory cytokine-1 "MIC-1"). Moreover, different pluripotency-associated transcription factors (Oct3/4, Nanog, Sox2 and Myc) and microRNAs that are involved in the epigenetic reprogramming and acquisition of stem cell-like properties by cancer cells during cancer progression may also be exploited as molecular biomarkers to predict the risk of metastases, systemic treatment resistance and disease relapse of cancer patients.

Introduction

Significant advancement in basic and clinical oncology during the last few years has led to earlier diagnosis and more effective therapeutic management of patients with leukemias and organ-confined tumors in the clinics (1-3). Although the surgical tumor resection may result in some cases to a complete remission, the rapid cancer progression of aggressive cancers to locally invasive and metastatic stages is generally associated with the development of resistance mechanisms by cancer cells to current anti-hormonal, radiation and/or chemotherapeutic treatments and disease relapse (1-3). At the present time, the metastatic cancers remain the leading cause of the death of cancer patients. Therefore, many research efforts have been made to identify and validate novel molecular biomarkers and therapeutic targets in cancer cells at primary and secondary tumors to prevent cancer progression and metastases and optimize the genetic- and proteomic-based individualized treatments of cancer patients (Fig. 1) (4-28).

Importantly, accumulating lines of evidence have revealed that the shedding of cancer cells from the primary tumors into the lymphatic vessels and peripheral circulation can occur very early during the cancer development and be dependent of cellular origin, genetic alterations and aggressiveness of cancer subtypes (16,29-41). Hence, some patients that undergo a complete surgical tumor resection with negative margins may show the presence of circulating tumor cells (CTCs) in the peripheral blood and disseminated tumor cells at the regional lymph nodes and distant tissues and organs (Fig. 1) (16,29-41). Consequently, CTCs that are able to survive in the bloodstream and spread at distant sites can persist and contribute to metastases and disease relapse even after an effective and apparently curative surgical resection of the primary tumor. In this regard, a growing body of experimental evidence has also revealed that cancer stem/progenitor cells endowed with stem cell-like properties, also designated as cancer-, tumor- and metastasis-initiating cells, can provide critical functions for tumor growth, metastases at near and distant tissues and

organs, treatment resistance and disease relapse. In fact, it has been shown that the most cancers may originate from the malignant transformation of immature tissue-resident stem/progenitor cells or their early differentiated progenies endowed with a high self-renewal ability and aberrant differentiation potential (2,42-44). The cancer stem/progenitor cells expressing specific stem cell-like markers such as CD133, CD44^{high}, nestin, aldehyde dehydrogenase (ALDH^{high}) and high levels of ATP-binding cassette (ABC) multidrug transporters have also been identified and isolated from primary and secondary neoplasms, including leukemias, melanomas, brain tumors and the most epithelial cancers and cancer cell lines (9.17,24,44-76). It has been shown that cancer stem/progenitor cells were able to give rise to the total tumor cell mass, including differentiated cancer cells that reconstituted the histological architecture and molecular characteristics of primary and secondary tumors closely resembling to original patient's tumors *in vivo* (9,17,45-57,59-66,68,69,71,77). Moreover, the data from recent studies have indicated that cancer stem/progenitor cells may be more resistant than their differentiated progenies to current anti-hormonal, radiation and chemotherapeutic treatments and targeted therapies (17,22-25,44,52,53,56-64,68,70,72,77-94).

We review here recent advances on the characterization of gene products often altered in cancer stem/progenitor cells and their differentiated progenies during primary cancer progression and dissemination through the peripheral circulation and metastases. The emphasis is on molecular gene signatures, epithelial-mesenchymal transition (EMT)- and stem cell-like biomarkers detected in cancer cells and exosomes associated with cancer progression, treatment resistance, disease relapse and poor outcome of cancer patients.

Heterogeneity of cancers

The most cancers are heterogeneous diseases and include some biological subtypes characterized by specific genetic and/or epigenetic alterations occurring in cancer stem/progenitor

cells and their differentiated progenies during cancer initiation and progression to locally invasive and metastatic stages (4,6,7,44,60,78,80,95-109). Although the molecular events that govern the spread of cancer stem/progenitor cells and their differentiated progenies at preferential metastatic sites remain not precisely defined, some lines of evidence suggest that particular gene signatures of cancer cells at primary cancers may be associated with their propensity at metastasizing at specific tissues and organs (4,6,7,95-102). More particularly, the alterations in the gene products involved in the acquisition of aggressive and migratory properties by highly tumorigenic cancer stem/progenitor cells during the EMT program at the primary tumor may result in their invasion to near lymph nodes and tissues, dissemination through the peripheral circulation and metastases at distant sites (Fig. 1) (42,90,91,96,97,110-127). Moreover, different gene products have also been identified in exosomes released by tumor cells and CTCs with stem cell-like features that may be used as diagnostic, prognostic and/or predictive biomarkers. In this regard, we review the gene products frequently altered in cancer cells, during cancer progression to locally advanced and metastatic states, exosomes and CTCs that have been associated with treatment resistance, disease relapse and poor overall survival of cancer patients.

Molecular gene signatures and stem cell-like biomarkers associated with aggressive and metastatic cancers

Some tissue-specific and whole genome approaches for molecular profiling of complex gene signatures of tumors and cancer cell lines have been developed in the few last years and led to the discovery of specific gene sets related to particular cancer types and which may predict the risk of metastatic dissemination at specific tissues and organs and overall outcome of cancer patients (Fig. 1) (4-15). Moreover, the *in vitro* and *in vivo* characterization of stem cell-like markers and functional features of cancer stem/progenitor cells in tumor tissue specimens from cancer patients

and cancer cell lines has also revealed their major implications in tumor growth, metastases, treatment resistance and disease relapse (Fig. 1) (15-27). We are reporting here the gene signatures and stem cell-like biomarkers of cancer cells associated with particular subtypes of aggressive cancers, including hormone-independent prostate and breast cancers.

Prostate cancer

Prostate cancer (PC) is among the most commonly diagnosed malignancies affecting men (128-130). Although an earlier diagnosis of PC patients has led to an improvement of their effective treatment by tumor resection surgery and/or radiation therapy, the locally advanced and metastatic castration-resistant prostate cancers (CRPCs) still represent the second leading cause of cancer-related death (128-130). In fact, anti-hormonal treatments and first-line docetaxel-based chemotherapies of metastatic CRPCs are only palliative and result in the death of most patients after about 12-19 months (128-130). Therefore, this inefficacy of current treatments against CRPCs underlines the importance to identify novel biomarkers to predict the risk of propagation to CRPCs. Of clinical relevance, a 11-gene signature related to the activation of polycomb-group protein BMI-1 pathway, which provides critical functions for the self-renewal of diverse normal and cancer stem cells, has notably been identified through a comparative genomic approach using primary and metastatic tumor specimens from transgenic adenocarcinoma of the mouse prostate (TRAMP) model and PC patients (4). The 11-gene signature includes 8 up-regulated genes (Ki67, cyclin B1, gastrulation brain homeobox 2, budding uninhibited by benzimidazoles 1 "BUB1" that acting as serine/threonine-protein kinase, kinetochore associated 2, ubiquitin-specific protease 22, host cell factor 1 and ring finger protein 2) and 3 down-regulated genes (ankyrin 3, fibroblast growth factor receptor 2 and carboxylesterase 1) (4). This 11-gene signature, which is associated with an up-regulation of stem cell-resembling expression profile of the BMI-1-regulated pathway, has been

shown to be a powerful predictor of PC progression to locally invasive and metastatic stages (4). The analyses of this 11-gene signature of BMI-1-driven pathway in 1153 clinical tumor specimens from cancer patients diagnosed with 11 different types of cancer have also indicated that the expression of these gene products was associated with a short interval to disease recurrence, distant metastases and death after therapy of cancer patients (4). These cancer types comprise acute myeloid leukemia, lymphoma, mesothelioma, medulloblastoma, glioma, and prostate, breast, lung, ovarian and bladder epithelial cancers (4). Importantly, high expression levels and/or activities of stem cell-like markers such as CD133, CD44, integrin-α_2, nestin, ALDH1 and CD49f in primary prostatic adenocarcinomas and bone metastases from patients and PC cell lines have also been associated with their aggressive and invasive phenotypes and treatment resistance (17-26,28). Particularly, the data from immunohistochemical analyses of integrin-α_2, integrin-α_6 and hepatocyte growth factor receptor (MET) in PC cells have revealed that an increase of expression levels of these markers in prostatic adenocarcinoma specimens from PC patients was associated with an enhanced risk of local invasion and bone metastasis (131). It has also been reported that several ALDH isoforms were expressed in specimens of primary prostate tumors with matched bone metastases and PC cell lines, and ALDH^{high} PC3M-Pro4 cells rapidly formed primary prostatic tumor and metastasized at distant sites *in vivo* as compared to ALDH^{low} PC3M-Pro4 cells (18,19). Of therapeutic interest, the knockdown of ALDH7A1 expression in PC3M-Pro4 cells has also been observed to result in a decrease of the integrin-α_2^{high}/integrin-α_v^{high}/CD44$^+$ PC3M-Pro4 stem/progenitor cell subpopulation and gene products involved in the EMT process and invasion, including snail, snail2, twist and extracellular molecule, osteopontin (19). Moreover, the ALDH7A1 silencing in PC3M-Pro4 cells significantly inhibited their clonogenic and migratory abilities *in vitro* as well as their capacity to form bone metastases *in vivo* (19). In the same way, it has also been observed that prostate-specific antigen (PSA$^{-/lo}$) PC cells from LNCaP, LAPC4 or LAPC9 cell lines

or tumor specimens from PC patients expressing stem cell-like markers such as ALDH, CD44, integrin-$\alpha_2\beta_1$ and with a long-term tumor-propagating ability were able to give rise to the total mass of PSA$^+$ PC cells *in vivo* (17). It has also been noted that PSA$^{-/lo}$ LAPC9 cells were resistant to androgen deprivation in castrated hosts, and high grade and recurrent prostatic adenocacinoma specimens of patients were enriched in PSA$^{-/lo}$ PC cells (17). These observations suggest that PSA$^{-/lo}$ PC cells may play critical functions for the tumor re-growth after anti-hormonal treatment initiation.

In addition, we have observed that sonic hedgehog (SHH/GLI-1), epidermal growth factor receptor (EGFR), pAkt, nuclear factor-kappaB (NF-κB) and macrophage inhibitory cytokine-1 (MIC-1) are frequently overexpressed in PC cells, including CD133$^+$ PC stem/progenitor cells, detected in prostatic adenocarcinoma and bone metastasis specimens from PC patients (23,24). The high expression levels and/or activities of these oncogenic products were also detected in the side population (SP) from tumorigenic and invasive WPE1-NB26 cells and metastatic PC3M-LN4 endowed with a high prostasphere-forming ability (23-25). Moreover, the targeting of hedgehog, EGFR, pAkt, NF-κB or MIC-1 by using cyclopamine, gefitinib, Akt inhibitor III, partenolide or anti-MIC-1 antibody was effective at inducing the apoptotic effects on SP and non-SP cells from WPE1-NB26 and PC3M-LN4 cell lines and improving cytotoxic effects induced by docetaxel on these SP and non-SP cells (23,24). Recent studies have also revealed that the changes in the expression levels and activities of hypoxia-inducible factors (HIFs) and microRNAs (miRNAs), which are short non-coding RNA molecules of about 22 nucleotides that are involved in the stringent control of normal stem cell behavior often occur in cancer cells including PC cells (27,28,132,133). HIFs and miRNAs may be involved in the epigenetic reprogramming and acquisition of stem cell-like features by cancer cells (27,28,132,133). More specifically, the up-regulation of HIF-1α and HIF-2α has been associated with the increased expression of pluripotency-

associated transcription factors (Nanog, Oct4 and Sox2) and EMT program-associated molecules in PC cells under normoxic and hypoxic conditions during PC progression to locally invasive and metastatic stages (27,28). The down-regulation of some tumor-suppressor miRNAs such as miR34a, let-7b, miR-106a and miR-141 combined with an up-regulation of miR-301 and miR-452 has also been observed to regulate the proliferation and tumorigenicity of CD133/CD44/integrin-$\alpha_2\beta_1$ PC stem/progenitor cell population and SP cells *in vitro* and *in vivo* (132,133).

Breast cancer

Breast cancer is among the most common cancers in women (128,134,135). Although a high survival rate is observed for early-stage breast cancer patients treated by tumor resection surgery and adjuvant treatments, the propagation to locally invasive and metastatic disease state is generally associated with the development of resistance mechanisms by cancer cells to current anti-hormonal treatments, targeted therapies, radiation and chemotherapies (128,134,135). Typically, breast cancer is a complex and highly heterogeneous disease that encompasses distinct cancer subtypes characterized by the malignant transformation of the basal and/or luminal breast epithelial cells in the mammary gland (136,137). In this regard, the data from analyses of the expression levels of gene products in breast cancer cells, including breast cancer stem cells (BCSCs) with stem cell-like features, have indicated that breast cancer subtypes may exhibit cellular and functional heterogeneities (5-14,138,139). It has been observed that CD44^{high}/CD24^{−/low} BCSCs are frequently detected in basal-like subtypes of breast cancer while these immature cancer cells are very low in erbB2/HER2^{+} breast tumors (8). Moreover, a transcriptional analysis of basal-like breast cancer cell lines has led to the identification of two major cancer subclasses in which basal B appeared mesenchymal-like, whereas basal A may have either a luminal-like or basal-like morphology. It has been observed that a CD44^{high}/CD24^{low} epithelial basal A cell subpopulation isolated from triple-

negative HMT3909S13 breast cancer cell line exhibited cancer stem cell features such as mammosphere formation and resistance to the standard chemotherapy as well as tumor-initiating capacity *in vivo* (6). In contrast, CD44^{high}/CD24^{-} mesenchymal-like basal B cell fraction does not exhibit these functional features (6). The data from comparative quantitative proteomic and gene array analyses of CD44^{high}/CD24^{low} BCSCs with an epithelial-like morphology have also indicated that these immature cancer cells expressed lipolysis-stimulated lipoprotein receptor, RAB25, S100A14 and mucin 1 (MUC1) (6). These CD44^{high}/CD24^{low} BCSCs also expressed a novel 31-gene signature that was related at the presence of distant metastases in estrogen receptor negative (ER^{-})-human breast cancer specimens (6). Conversely, the results from another investigation have revealed that CD44^{high}/CD24^{low} BCSCs that were detected in patient's primary tumors exhibited a gene signature as the claudin-low molecular subtype which is characterized by the expression of several EMT program-associated molecules (10). It has been noted that the CD44^{high}/CD24^{low} BCSCs expressing mesenchymal markers were enriched in patient's primary tumors after either endocrine letrozole-based therapy or docetaxel-based chemotherapy (10). These findings suggest that breast cancer cells endowed with an epithelial or mesenchymal phenotype can exhibit cancer stem cell properties dependent of breast cancer subtypes and be involved in the metastasis formation and treatment resistance.

On the other hand, the data from analyses of global gene expression profiles have also indicated that a common subset of 81 genes was differentially expressed between ER^{-} and ER^{+} breast tumor specimens and breast cancer cell lines as well as in androgen-independent *versus* androgen-sensitive PC cell lines (11). It has been noted that some signaling elements of EGFR pathway were enriched in ER^{-} breast and androgen-independent PC cell lines suggesting that the sustained activation of this tumorigenic cascade may contribute to the growth and survival of these hormone-independent cancer cells (11). Interestingly, it has been reported that an 8-gene signature (EGFR, integrin-α_3,

myosin light chain kinase, retinoic acid induced protein 14, AHNAK nucleoprotein, glutaminase, interleukin-32 and nicotinamide N-methyltransferase) was associated with the invasion and sensitivity of a panel of 78 tumor cell lines to chemotherapeutic drugs, including paclitaxel, docetaxel, erlotinib, everolimus and dasatinib (14). This set of eight genes also predicted the relapse-free survival for breast cancer patients (14).

Other cancer types

Recent investigations have also led to the discovery of specific gene signatures and potential biomarkers in cancer tissue specimens for improving the personnel management of patients with other aggressive cancers such as brain, melanoma, squamous cell carcinoma (ESCC) and lung, pancreatic, colorectal, esophageal and ovarian cancers (Table 1) (3,14,70,140-150). For instance, the real-time PCR data from gene expression profiles of brain tumor stem-like cells (BTSCs) from 5 human glioblastoma multiforme (GBMs) and matched GBMs tissue specimens have indicated that SHH and GLI1 hedgehog signaling elements were co-expressed only at high levels in BTSCs 1-3 and not in BTSCs 4 and 5 (142). It has also been noted that the SHH/GLI1 cascade-induced BTSC proliferation *via* PI3K/Akt/molecular target of rapamycin (mTOR), was inhibited by small interfering RNA (shRNA) against human GLI1 or rapamycin *in vitro* and in an intracranial tumor model in athymic mice (142). Of clinical interest, higher expression levels of SHH, patched receptor 1 (PTCH1) and GLI1 expression have also been detected in phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-expressing brain tumors than in PTEN-deficient brain tumors and associated with a poorer survival time of GBM patients (142). Furthermore, it has been shown that melanoma cells from patient melanoma specimens expressing high levels of ALDH1A1 and ALDH1A3 isoforms dislayed a high self-renewal ability, were more resistant to chemotherapeutic agents and more tumorigenic than ALDH$^-$ melanoma cells in non-obese diabetic (NOD)/severe

immonodeficient (SCID) mice (140). It has also been noticed that the silencing of the ALDH1A gene by shRNA led to an arrest in cell cycle and apoptosis of ALDH1A^{+} melanoma cells, inhibition of tumor growth *in vivo* and sensitized the ALDH1A^{+} melanoma cells to cytotoxic effects of chemotherapeutic drugs (140). These findings implicate that ALDH1A and ALDH3A isozymes are putative biomarkers for melanoma stem cells and constitute attractive therapeutic targets for treating melanoma patients. In the same way, human pancreatic ductal adenorcinoma (PDAC) specimens have also been observed to contain a SP subpopulation exhibiting the gene expression signatures that are associated with pancreatic cancer stem cell-like characteristics and resistance to chemotherapeutic drugs (145). Among these genes, expression levels of ABCB1 multidrug transporter and CXC chemokine receptor 4 (CXCR4) were correlated with a worse survival of pancreatic cancer patients (145).

Altogether, these studies have led to the identification and validation of gene expression signatures and potential diagnostic, prognostic and predictive biomarkers related to stem cell-like cancer cells, EMT program, treatment resistance and relapse of different aggressive cancers. Future investigations are however necessary to validate the expression of these gene signatures and their implications in the treatment resistance on larger cohorts of tumor tissue specimens from cancer patients. In this regard, recent studies have also revealed the possibility to analyze the phenotypic features of exosomes secreted by cancer cells and CTCs, including circulating cancer stem/progenitor cells, to assess the tumor stage and risk of cancer progression, metastases and disease relapse of cancer patients.

Molecular biomarkers of exosomes

Accumulating lines of evidence have indicated that exosomes that are secreted by normal and cancer cells can act as key mediators in signal transduction mechanisms that are involved in short- and long-distance cell-to-cell communication (151-158). In fact, the exosomes are small microvesicles (sized about 50-100 nm) derived from cellular endocytosis that may contain specific lipids, proteins, mRNAs and miRNAs encapsulated in a cholesterol-rich phospholipid membrane (151-158). More particularly, the exosomes that are released by cancer cells in their surrounding microenvironment and peripheral circulation may adhere *via* their antigenic surface molecules or transfer their cytoplasmic contents through membrane fusion to their targeted host cells, including other cancer cells, stromal cells, endothelial cells and immune cells (Fig. 1) (151-158). Hence, the shedding of exosomes by cancer cells under normoxic and hypoxic conditions may modulate immune system and promote the malignant behavior of recipient cells in their tumor microenvironment and angiogenesis as well as the formation of pre-metastatic niches, metastases at distant tissues and organs and treatment resistance (Fig. 1 and Table 2) (151-161). Importantly, recent advances in the development of isolation techniques of exosomes from some types of body fluids or cancer cell culture supernatants using centrifugation, ultrafiltration, immunoprecipitation, magnetic activated cell sorting (MACS) and immunoaffinity capture methods have led to an improvement of the purity of exosomal preparations (151-160,162-165). Hence, several studies carried with these isolation methods have indicated the presence of exosomes in blood, plasma, serum, lymph, urine, cerebrospinal fluid or ascites from patients with melanoma and brain, ovarian, breast, pancreatic, colon, bladder, prostate and lung cancers as well as conditioned medium of different cancer cell types (Fig. 1 and Table 2) (151-160,162-165). It has also been observed by proteomic profiling that different exosomal preparations of different cancer types frequently expressed a set of biomarkers. The common biomarkers of most exosomes include tubulin, actin,

tetraspanins (CD9, CD63, CD81 and CD82), Alix, TSG101, clathrin, small GTPases (Rab proteins), annexins, heat-shock proteins (Hsp70 and Hsp90), 14-3-3 proteins and MHC class I molecules that are involved in the biosynthesis, cytoskeletal structure and trafficking of exosomes (160,163-165). Furthermore, tumor cell-derived exosomes also harbored specific RNAs, miRNAs and proteins, including stem cell-like markers (CD133, CD44 and/or CD105) and oncogenic products (EGFR, EGFRvIII mutant, erbB2/HER2/Neu, MET, claudins and/or FASN) that are related to cancer progression, metastases and treatment resistance (151-160,162-167). For instance, the data from proteomic analyses of highly purified exosomes derived from two ovarian cancer cell lines, OVCAR-3 and IGROV1, have indicated that several proteins of tumorigenic pathways that are overexpressed in ovarian cancer tissues and associated with migration, invasion, immune modulation, angiogenesis and metastasis were also detected in exosomes (163). These oncogenic products comprise EGFR, epithelial cell surface antigen (EpCAM), proliferation cell nuclear antigen (PCNA), tubulin β-3 chain (TUBB3), apolipoprotein E (APOE) and claudin 3 (CLDN3) (163). Moreover, the exosomes expressing T cell receptor (TCR), CD20, HLA-DR, B7-2, HER2, CA125 and histone H2A have been detected in 85.4% (35/41) of ascites samples from ovarian cancer patients (168). It has also been shown that the exosomes induced the apoptosis of precursor and mature dendritic cells (DCs) and peripheral blood mononuclear cells (PBMCs) and impaired the cytotoxic activity of PBMCs in the presence of DCs suggesting their implication in the modulation of anti-tumor immunity (168).

In addition, the tumor-specific and constitutively active form of EGFR designated as EGFRvIII mutant has also been detected in serum exosomes from 7 out of 25 glioblastoma patients (153). It has been noted that EGFRvIII protein expressed by glioma cells may be transferred *via* exosomes to other glioma cells lacking EGFRvIII, and thereby promoted their oncogenic transformation *via* the activation of phosphatidylinositol-3' kinase (PI3K)/Akt and mitogen-activated protein kinase

(MAPK) pathways (Fig. 1 and 2A and Table 2) (154). These molecular events induced the changes in the expression of EGFRvIII-regulated genes such as vascular growth factor "VEGF", Bcl-xL and p27 in host glioma cells and increased their anchorage-independent growth capacity (154). It has also been observed that exosomes released from glioblastoma tumor cells also contained mRNA, miRNA and angiogenic proteins that could be transferred to host cells, including normal brain microvascular endothelial cells, and thereby stimulate the tubule formation (154). In the same way, cancer stem cells expressing the mesenchymal stem cell marker CD105 from human renal cell carcinoma also released exosomes that contained pro-angiogenic mRNAs and miRNA (169). It has also been noted that the exosomal mRNAs and miRNA in turn activated the growth of normal endothelial cells and induced the vessel formation and angiogenesis after implantation in SCID mice (169). Moreover, the treatment of SCID mice with exosomes from CD105-positive cells was effective at promoting the formation of lung metastases induced by intravenous injection of renal carcinoma cells (169).

Importantly, several lines of experimental evidence have also indicated that the exosomes may be involved in the formation of pre-metastatic niches and transfer of more aggressive and drug-resistance phenotypes to cancer cells and modulation of treatment responses of cancer patients (Table 2) (151,170-176). For instance, CD133-expressing exosomes purified from FEMX-I cell line, which was derived from a lymph node metastasis of a patient with malignant melanoma, have been observed to express several pro-metastatic proteins, including CD44, MAPK4K, GTP-binding proteins, ADAM10 and annexin A2 and high levels of miRNAs with cancer-related functions (174). It has been shown that primary melanoma-derived exosomes can induce the recruitment and growth of melanoma cells to sentinel lymph nodes *in vivo* by inducing extracellular matrix deposition and vascular proliferation in the lymph nodes (175). Moreover, the exosomes from highly metastatic melanomas also contributed to the pre-metastatic niche formation through the up-regulation of the

MET oncoprotein in CD45$^-$/KIT$^{low/+}$/tyrosine kinase with immunoglobulin-like and EGF-like domains-2 (TIE2$^+$) bone marrow-derived progenitor cells (Fig. 1) (170). The enhanced expression of MET by bone marrow progenitor cells resulted in their reprogramming toward pro-vasculogenic and pro-metastatic phenotypes (170). The gene signature of exosomes released by melanoma, including MET, tyrosinase-related protein-2, very late antigen-4 , HSP70 and HSP90, has also been associated with a poor prognosis of melanoma patients (Table 2) (170). Moreover, it has been reported that the exosomes isolated from serum samples of PC patients promoted the proliferation and invasion of PC cell lines while the exosomes from age-matched healthy controls have not significant effects (151). The exposure of DU145 and 22Rv1 PC cells to exosomes from serum samples of PC patients treated with docetaxel was also effective at enhancing their docetaxel resistance as compared to exosomes from untreated PC patient's sera (151). It has also been noticed that docetaxel-resistant DU145RD and 22Rv1RD cell lines secreted the exosomes expressing P-glycoprotein, which could be transferred and conferred docetaxel resistance to parental DU145 and 22Rv1 cell lines (Fig. 1) (151). The PCR-based analyses of urine samples from PC patients have also indicated that the transcripts of prostate cancer gene-3 (PCA-3) and TMPRSS2;ERG gene fusion were detected in urinary exosomes suggesting their potential use as a non-invasive method for the diagnosis and follow-up of PC patients (162). Moreover, it has also been shown that the exosomes released by SKBR3 breast cancer cells overexpressing HER2 oncoprotein interfered with the anti-tumoral activity of the anti-HER2 antibody, trastuzumab (Herceptin®) (173).

Therapeutic applications of exosomes

The molecular targeting of tumor cell-derived exosomes by interfering with their biosynthesis, secretion and and/or interactions with cell antigens or receptors also represents a promising approach to prevent the cancer progression, pre-metastatic niche formation and metastases at distant

sites (Fig. 2) (161,170,177-180). For example, the targeting of neutral sphingomyelinase 2, which is involved in the ceramide biosynthesis and up-regulation of exosome secretion, with chemical drug GW4869 has been shown to inhibit the lung metastases in lung cancer cell-bearing mice *in vivo* (177). Moreover, the interference with molecular pathways of exosome secretion by short hairpin RNA (shRNA) silencing of small GTPase Rab27a or Rab27b or two Rab27 effectors, known as Slp4 and Slac2b, also inhibited the exosome secretion by cervical adenocarcinoma Hela cells, 4T1 and TS/A mammary carcinoma cells and melanoma cells (170,178,179). It has also been noted that Rab27a blockade resulted in decreased primary tumor growth and lung dissemination of a metastatic carcinoma (4T1), but not of a nonmetastatic carcinoma (TS/A) (178). In A431 human tumor xenografts in mice, angiogenic endothelial cells stain positively for human EGFR and phospho-EGFR, while treatment with annexin homodimer, diannexin which cloak phosphatidylserine residues on the surfaces of exosomes led to a reduction of tumor growth rate and microvascular density (Fig. 2A) (161). Of particular clinical interest, a novel medical device using an affinity plasmapheresis platform termed as Aethlon ADAPT™ system has also been shown to be effective to perform extracorporeal hemofiltration and elimination of tumor cell-derived exosomes from the entire circulatory system (Fig. 2B) (180).

Recent investigations have also revealed that exosomes could be used as natural cell-derived membrane vehicles for systemic delivery of therapeutic agents interfering with the functions of tumor-specific mRNAs, miRNAs and oncogenic products in target cells as well as immune regulatory tools in cancer immunotherapy (Fig. 2C) (181-184). As a matter of fact, exosomes engineered to deliver spherical nucleic acid constructs into PC3 prostate cancer cells down-regulated miR-21 target by approximately 50% (183). Furthermore, it has been shown exosomes that are released by tumor cells and dendritic cells may be used to elicit specific immune responses against established tumors (181,182). For instance, the data from a Phase I clinical trial have

indicated that a combination of autologous ascites-derived exosomes with granulocyte macrophage colony-stimulating factor (GM-CSF) was safe and well tolerated and induced tumor antigen-specific cytotoxic T-cell responses without side effects in patients with advanced colorectal cancer (181).

Together these data suggest important roles of exosomes secreted by cancer cells in their surrounding microenvironment in the acquisition of a more malignant behavior by host cancer cells and promoting immune escape and tumor neovascularization during cancer progression. Moreover, the exosomes can play critical functions in the establishment of pre-metastatic niches, recruitment and homing of cancer cells at distant tissues and organs, metastases and treatment resistance. Hence, these results support the interest to use the exosome-specific biomarkers as new diagnostic, prognostic and predictive indicators and to develop new exosome-based targeted therapies for treating aggressive and metastatic cancers.

Molecular biomarkers of circulating tumor cells

The enumeration of CTCs isolated from whole blood samples of patients with metastatic cancers before or post-treatment initiation and characterization of their molecular biomarkers may constitute a non-invasive and real-time method for improving accuracy of diagnosis and prognosis and choice of their therapeutic management, including use of appropriate targeted therapy (16,31-35,185). Several strategies, including CellSearch[®] platform, microfluidic devices and cell size-based separation methods, have been developed to detect, enrich and isolate rare CTCs from peripheral blood samples (Fig. 1 and Table 3) (16,31-34). These detection techniques involve the CTC immunocapture from peripheral blood samples using different specific antibodies or size-based filters (Fig. 1 and Table 3) (16,31-34). More particularly, the CellSearch® technology, which

consists to use immunomagnetic beads coated with an antibody directed against EpCAM molecules, can allow researchers to isolate epithelial cancer cells expressing EpCAM surface marker (16,31,33-35,185,186). In a second step, the analyses of different biomarkers of the interest expressed by captured CTCs can be performed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using DNA extracted from CTCs or immunofluorescence staining of CTCs (Fig. 1) (16,31,34,185). The biomarkers of CTCs include stem cell-like markers (CD133, CD44, ALDH and ABC multidrug transporters), cytokeratins, EGFR, EGFRvIII, HER2 and K-Ras as well as the absence of the leukocyte-specific marker CD45 (Table 3) (16,31,34,185). Hence, it has been observed that the number and phenotypic features of CTCs captured using CellSearch® assay, microfluidic CTC-ships and other detection techniques given significant information on the treatment benefit and helped to predict the overall outcome of patients with locally advanced and metastatic breast, prostate, ovarian, pancreatic, lung, colorectal and pancreatic cancers (Table 3) (16,31-35,37,185,187-191). For instance, the characterization of CTCs from patients with metastatic CRPC has indicated that the majority of CTCs co-expressed epithelial proteins (EpCAM, cytokeratins and E-cadherin), mesenchymal markers (vimentin, N-cadherin and O-cadherin) and the stem cell-like marker CD133 (34).

In addition, the results of qRT-PCR analyses of CTCs from breast cancer patients have also indicated a direct relationship between the expression of stem cell-like markers such as Nanog, Oct3/4, Sox-2, nestin and CD34 on CTCs and the stage of the disease progression (36). Moreover, the analyses of blood samples from 502 breast cancer patients with the AdnaTest BreastCancer Select for the detection of EpCAM, MUC1, HER2 and β-actin transcripts have revealed that CTCs were detected in 19% of samples, and 29% samples expressed at least one of EMT markers (twist1, Akt2 and PI3Kα) while ALDH1 stem cell-like marker was detected in 14% of the samples (38). In

another study, CTCs were also detected in 31% of 226 blood samples from 39 metastatic breast cancer patients and 62% were positive for at least one of EMT markers and 69% for ALDH1 (39). It has also been noticed that the expression of EMT markers and ALDH1 was detected in 62% and 44% of breast cancer patients that were non-responders to the therapy while only 10% and 5% of the responders expressed these markers, respectively suggesting their potential use as predictive biomarkers (39).

More recently, single CTCs and multicellular clusters in a viable state have also been isolated from bloodstream of patients with metastatic lung cancers by dean flow fractionation (40). This size-based separation method may overcome the problems associated with the immunocapture with antibodies that is dependent of antigens expressed by CTCs (40). The number of CTCs detected by dean flow fractionation in whole blood samples from 20 metastatic lung cancer patients was 39.1 ± 24.8 CTCs/mL while CTCs in blood samples from 20 healthy individuals was negligible (0.79 ± 0.42 CTCs/mL) (40). It has also been observed that the CTC population detected in blood samples from advanced lung cancer patients contained cells co-expressing cytokeratin and CD133 stem cell-like marker (40). Moreover, the qRT-PCR analyses of peripheral blood samples from patients with primary melanoma (early stages, 0-II, n=154) and metastatic melanoma (late stages, III-IV, n=76) have indicated that all samples contained CTCs (41). It has also been noted that melan-A known as melanoma antigen and stem cell-like marker, ABCB5 multidrug transporter were expressed in 45% and 49% of patients showing melanoma recurrence after treatment initiation, respectively (41).

The use of specific biomarkers of CTCs, including stem cell-like markers, represents promising strategy for improving the diagnosis, prognosis and therapeutic management of patients with aggressive and metastatic cancers.

Conclusions and Perspectives

The characterization of gene products expressed in tumor tissue specimens and CTCs from cancer patients and exosomes released by cancer cells has led to the identification and validation of some gene signatures and potential stem cell-like markers. These altered gene products could be used as non-invasive diagnostic, prognostic and predictive tools in conjunction for monitoring the risk of tumor progression and metastases and determining the choice of therapeutic treatments of cancer patients. Furthermore, the molecular targeting of specific markers of cancer stem/progenitor cells, CTCs and exosomes also represents a potential strategy could prevent cancer progression and metastases and overcome the treatment resistance and disease relapse of cancer patients.

Future investigations are however required to optimize the sensitivity and reproducibility of immunocapture methods used for the enrichment and enumeration of CTCs and circulating exosomes from whole blood samples in order to consider their low number, potential heterogeneity and specific biomarkers. Indeed, the detection and purification of rare CTCs, including circulating cancer stem cells, and circulating exosomes may be obscured by the non-specific background of other hematopoietic cell types and overwhelming material in whole blood samples. Therefore, the optimization of *in vivo* detection platforms suitable for a continuous monitoring of whole blood volume could markedly improve the probability to detect rare CTCs, including circulating cancer cells with stem cell-like features. Moreover, the use of additional antibodies directed against epithelial, mesenchymal and/or stem cell-like surface markers of CTCs could be included in immunoaffinity capture methods, such as CellSearch platform and microfluidic ships, to detect the EpCAM-negative CTCs or exosomes. It will also be of interest to further establish the relationship between the expression levels of EMT process- and stem cell-like markers detected on captured CTCs and exosomes and the stage of tumor progression, treatment response and survival rate using a large cohort of cancer patients. Hence, the combined use of these new biomarkers detected in

primary and metastatic tumor tissues, CTCs and exosomes should lead to the development of multiplex biomarker approaches for improving the accuracy of current diagnostic and prognostic tests and efficacy of individualized treatments for cancer patients. Of clinical relevance, the development of new nanotheranostic platforms for the combined *in vivo* detection by imaging and nanobubble-based targeted therapies of CTCs and circulating exosomes also may constitute a promising approach for real-time diagnosis and treatment of patients with aggressive and metastatic cancers.

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Figure legends

Fig 1. Schematic representation of functions of cancer stem/progenitor cells during cancer progression and metastasis and characterization of their biomarkers. The scheme shows cancer stem/progenitor cells endowed with stem cell-like properties and which can generate the total cancer cell population at the primary and secondary tumors. Moreover, the exosomes released by cancer cells, which may contribute to the malignant transformation of other cancer cells *via* the transfer of oncogenic products and drug resistance-associated molecules such as EGFRvIII and P-glycoprotein, is also illustrated. The possibility to perform the characterization of molecular gene signature and biomarkers of cancer cells, exosomes and CTCs, including cancer stem/progenitor cells expressing stem cell-like markers, is also indicated.

Fig 2. Schematic representation of functions of exosomes released by cancer cells in cancer progression, metastasis, angiogenesis and treatment resistance and their potential therapeutic applications. The scheme shows **A.** the oncogenic molecules such as EGFR or EGFRvIII, which can be shed from cancer cells *via* exosome secretion and transferred to other adjacent cancer cells and\or endothelial cells. Hence, the activated EGFR or EGFRvIII may stimulate MAPK and Akt pathways and expression of their targeted genes, including VEGF, in host cancer cells and endothelial cells in their surrounding tumor microenvironment. VEGF released by endothelial cells in turn can activate in an autocrine manner the VEGFR2 cascade and tumor angiogenesis. Of therapeutic interest, the inhibition of exosome secretion from cancer cells carried by shRNA silencing of Rab27a/b, using an inhibitor of sphingomyelinase (GW4869) or annexin\diannexin and which represent potential strategies to prevent the promoting effects of exosomes on tumor progression, angiogenesis and metastasis is indicated. **B**. The elimination of tumor cell-derived

exosomes from the entire circulatory system which can be performed by extracorporeal hemofiltration using novel medical devices such as an affinity plasmapheresis platform designated as Aethlon ADAPT™ system is illustrate. **C.** The systemic delivery of therapeutic agents incorporated in cellular or synthetic exosomes and which are able to inhibit the functions of tumor-specific mRNAs, miRNAs such as spherical nucleic acid constructs and\or oncogenic products in target cancer cells is also illustrated.

Table 1. Characterization of molecular biomarkers detected in patient tissue specimens and their clinical significances in diverse aggressive and metastatic cancers

Cancer type	Biomarker detection method	Results and clinical significance		Ref.
		Number of patients/samples	Biomarkers and their diagnostic, prognostic and predictive potentials	
11 different cancer types including hematologic, brain and epithelial cancers	Microarray and qRT-PCR analyses	1153 clinical tumor specimens from cancer patients diagnosed with acute myeloid leukemia, lymphoma, mesothelioma, medulloblastoma, glioma, and prostate, breast, lung, ovarian or bladder epithelial cancers	An 11-gene signature that was associated with an up-regulation of stem cell-resembling expression profile of the BMI-1-regulated pathway has been shown to be a powerful predictor of prostate cancer progression and metastases. The expression of this 11-gene signature has also been associated with a short interval to disease recurrence, distant metastases and death after therapy of patients with 11 different cancer types.	(4)
Prostate cancer	Immunhistochemical staining	Tissue microarray (TMA) containing primary prostate tumor sections ($n = 30$) as well as 10 primary prostate tumors and their 10 matched bone metastases from PC patients	ALDH7A1 isoform was highly expressed in 83% clinical specimens of primary prostate tumors (TMA) as well as in 80% and 90% of PCs and their matched bone metastases, respectively. These data suggest that ALDH7A1 could constitute a potential biomarker for the stratification of PC patients at risk of developing metastatic disease.	(18)
Prostate cancer	Immunhistochemical staining of PSA$^+$ and PSA$^{-/lo}$ cells in PC sections	Primary tumor specimens from untreated patients with Gleason score 7 ($n = 10$) and 9 or 10 ($n = 10$), and treatment-failed patients ($n = 23$)	High grade and recurrent prostatic adenocacinoma specimens of patients were enriched in PSA$^{-/lo}$ PC cells suggesting that PSA$^{-/lo}$ PC cells with stem cell-like properties may play critical functions in the tumor re-growth after treatment initiation.	(17)
Prostate cancer	Immunhistochemical staining	76 Primary tumor specimens from PC patients with Gleason scores 6-10 plus 30 bone metastasis specimens from PC patients	The expression levels of EGFR, Ser^{473}-pAkt, NF-κB p65 and MIC-1 proteins were significantly enhanced in the same subset of 76 cases of PC specimens during the disease progression and detected in a small subpopulation of CD133$^+$ PC cells and the bulk tumor mass of CD133$^-$PC cells. All of these biomarkers were also overexpressed in 80-100% of 30 PC metastasis bone tissue specimens indicating their potential use as diagnostic and prognostic biomarkers.	(24)

Breast cancer	Analysis of a gene expression signature from CD44$^+$/CD24$^{-/low}$ cells and mammosphere-forming cancer cells in breast cancer specimens	The gene expression signature was analyzed in 36 tumors (18 luminal A/B, 13 basal-like, and 5 ErbB2-enriched). For therapeutic significance of signature, 18 breast cancer patient pairs before and after therapy with letrozole or 12 patient pairs before and after docetaxel therapy were used.	A gene expression signature common to both CD44$^+$/CD24$^{-/low}$ BCSCs and MS-forming breast cancer cells was mainly detected in patient's primary tumors as the claudin-low molecular subtype expressing several EMT program-associated molecules. The CD44$^+$/CD24$^{-/low}$-MS and claudin-low signatures were also enriched in patient's primary tumors after either endocrine letrozole-based therapy or docetaxel-based chemotherapy. These results support the potential application of the CD44$^+$/CD24$^{-/low}$-MS signature to predict the treatment resistance and relapse.	(10)
Breast and prostate cancers	Comparative analyses of profiles of hormone-sensitive and -independent breast cancers and PC cell lines with gene expression data sets from patient's breast cancers.	Breast ER-status signature was established from ER$^-$ (hormone-independent) and ER$^+$ (hormone-dependent) invasive breast cancer (n=295) profile data sets and 18 different breast cancer cell lines (ten of them ER-). The gene signature data sets from 8 androgen-sensitive and androgen-independent PC cell lines was also used.	A common subset of 81 genes was differentially expressed between ER$^-$ and ER$^+$ breast tumor specimens and breast cancer cell lines as well as in androgen-independent *versus* androgen-sensitive PC cell lines. EGFR signaling elements were also enriched in ER$^-$ breast and androgen-independent PC cell lines suggesting that the EGFR tumorigenic cascade may contribute to hormone-independent phenotypes of the breast cancer and PC cells.	(11)
Breast and lung cancers	Comparative analyses of invasion assay and Affymetrix gene expression data on a panel of cancer cell lines with chemo-sensitivity data.	Invasion-associated genes established from 60 human cancer cell lines developed by the National Cancer Institute were compared to chemo-sensitivity data of 99 anti-cancer drugs and clinical data from adjuvant chemotherapy cohorts in 508 breast and 71 lung cancer patients and a cohort of untreated patients (controls).	An 8-gene signature (EGFR, integrin-α$_3$, myosin light chain kinase, retinoic acid induced protein 14, AHNAK nucleoprotein, glutaminase, interleukin-32 and nicotinamide N-methyltransferase) was associated with the invasion and sensitivity of a panel of 60 tumor cell lines to paclitaxel, docetaxel, erlotinib, everolimus and dasatinib. The 8-gene signature also predicted the relapse-free survival for breast and lung cancer patients.	(14)

Non-small cell lung cancer (NSCLC)	Immunohistochemical analysis	Various histological tissue specimens of lung squamous cell carcinoma (SCCs) and lung adenocarcinomas in two independent large TMA sets (n1 = 287 and n2 = 511 were used.	A marked nuclear expression of SOX2 embryonic stem cell transcriptional factor was detected in all normal bronchial epithelia, alveolar bronchiolization structures and premalignant lesions in SCC development (hyperplasia, dysplasia and carcinoma *in situ*). In contrast, SOX2 protein expression was not seen in all normal alveoli and atypical adenomatous hyperplasias. Moreover, SOX2 expression was greatly higher in lung SCCs compared to lung adenocarcinomas.	(144)
Pancreatic cancer	Whole-genome expression analyses of side population (SP) cells and main non-side (Non-SP) cell fraction isolated from human pancreatic cancer resections specimens by FACS	SP cells were obtained from human pancreatic ductal adenocarcinoma (PDAC) specimens (n= 32) and the prognostic value of the SP gene signature was validated in a large independent series of PDAC patients (n = 78).	32 PDAC specimens contained a SP cell subpopulation exhibiting an up-regulation of genes associated with markers of putative pancreatic cancer stem and therapy resistance. The validation of SP cell-derived gene signatures of 32 or 10 up- or down-regulated genes in a different set of 78 PDAC samples has also indicated that the expression levels of ABCB1 multidrug transporter and CXCR4 were correlated with worse patient survival suggesting their potential used as prognostic biomarkers and therapeutic targets.	(145)
Brain cancer	The mRNA expression levels of PTEN, SHH, PTCH1, and GLI1 were determined by real-time PCR assays.	55 GBM tissue specimens of patients (age range= 29-75) who were diagnosed with *de novo* glioblastoma and treated with external beam radiation therapy to 60 Gy and chemo-therapy were used. The GBM samples were separated into a PTEN expressing group and a PTEN-deficient group on the basis of their PTEN expression.	The expression levels of SHH and GLI1 were significantly higher in PTEN-expressing GBM specimens than in PTEN-deficient GBMs. The higher expression levels of SHH, PTCH1, and GLI1 in PTEN-co-expressing GBM specimens was also associated with reduced survival of patients. These data indicate that hedgehog signaling elements may constitute potential prognostic biomarkers and molecular targets for individualized treatment of GBM patients overexpressing SHH/GLI1.	(142)
Melanoma	Analyses of ALDH activity by Aldefluor assay and gene expression by microarray analyses, qRT-PCR and immunohischemistry	3 primary and 6 metastatic tumors from melanoma patients	8 patient's tumors harbored small $ALDH^+$ sub-populations, ranging from 0.08% to 1.15%. The gene expression signatures of melanoma cells expressing high levels of ALDH1A and ALDH3A from patient's melanoma specimens were characterized by expression of some genes associated with stem cell functions.	(140)

Table 2. Potential applications of molecular biomarkers of circulating exosomes for the diagnosis, prognosis and prediction of treatment responses of diverse aggressive and metastatic cancers

Potential application of exosomes	Cancer type	Exosome source and purification and detection methods	Results and their significances	Ref.
Diagnostic biomarkers	Ovarian cancer	Tumor-derived exosomes were isolated from peripheral blood of 50 women diagnosed with serous papillary adenocarcinoma of the ovary (stages I-IV) using a MACS procedure with anti-EpCAM coupled to magnetic microbeads.	An 8 diagnostic microRNA signature (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214) was detected in ovarian tumor-derived EpCAM-exosomes and ovarian tumor specimens isolated from same cancer patients. In contrast, these exosomal microRNAs were not be detected in normal controls. Moreover, the levels of circulating exosomes detected in women increased with the stages I-IV of ovaian cancer.	(167)
Diagnostic biomarkers	Prostate cancer	Exosomes were prepared by differential centrifugationthe from urine samples of 11 PC patients.	The expression of PC biomarkers, including prostate cancer gene-3 (PCA-3) and TMPRSS2;ERG gene fusion, was detected in urinary exosomes suggesting their potential use for the diagnosis and monitoring of PC patients.	(162)
Diagnostic biomarker	Brain tumor	Exosomes were purified by differential centrifugation from serum samples from 25 GBM patients and 30 healthy individuals.	Tumor-specific and constitutively active EGFRvIII mutant was detected by RT-PCR in serum exosomes from 7 out of 25 (28%) GBM patients while EGFRvIII mRNA was not detected in serum exosomes from 30 normal individuals used as controls.	(153)
Diagnostic and prognostic biomarker	Esophageal squamous cell cancer (ESCC)	Exosomes were extracted from the sera samples of patients with ESCC and benign diseases without systemic inflammation.	The exosomal miR-21 expression was significantly up-regulated in serum samples from patients with ESCC *versus* benign diseases without systemic inflammation. Exosomal miR-21 positively correlated with tumor progression and aggressiveness, lymph node status and the presence of metastasis with inflammation and clinical stage without inflammation.	(166)
Diagnostic and prognostic biomarkers	Melanoma	Exosomes were purified by centrifugation from peripheral blood samples	An exosome-specific melanoma signature comprised of MET, tyrosinase-related protein-2, very late antigen-4, HSP70 and HSP90 has been associated with stage and outcome of melanoma patients.	(170).

|		from stage I-IV melanoma subjects.		
Predictive biomarker	Breast cancer	Exosomes were isolated from HER2-overexpressing breast carcinoma cell lines SKBR3 and BT474 or breast cancer patient's serum samples.	Exosomes from HER2-positive SKBR3 and BT474 tumor cell-conditioned supernatants or breast cancer patients' serum expressing HER-2 have been observed to interact with humanized antibody trastuzumab. Moreover, the data from functional assays have also revealed that exosomes suppressed the inhibitory activity of trastuzumab on the SKBR3 cell proliferation while lapatinib had no effects. These findings suggest that HER2-positive exosomes can modulate the sensitivity of breast cancer cells to trastuzumab, and consequently HER2-driven tumor aggressiveness.	(173)
Diagnostic and predictive biomarker	Prostate cancer	Exosomes were purified by ultracentrifugation from plasma and serum samples from 39 PC patients, 20 benign prostatic hyperplasia (BPH) patients, 8 recurrent PCs and 16 healthy controls.	Survivin level was significantly up-regulated in the tumor-derived exosomes compared to those from BPH and controls. Exosomal survivin levels was also higher in patients that had relapsed after chemotherapeutic treatment compared to controls.	(172)
Diagnostic, prognostic and predictive biomarkers	Brain cancer	Profiling of circulating exosomes labeled with target-specific magnetic nanoparticles was directly performed in blood samples of 24 GBM patients and 8 healthy volunteers using microfluidic chip. Exosomes were detected with miniaturized nuclear magnetic resonance system	Data of protein profiling of circulating exosomes have indicated that the expression of a combination of four markers (EGFR, EGFRvIII mutant, podoplanin and cytosolic isocitrate dehydrogenase 1) considerably improved the detection accuracy (> 90%) as compared to a single marker (< 76). Circulating exosome profiling also predicted treatment outcomes of GBM patients and differentiate between responders and non-responders to a treatment with alkylating agent, temozolomide plus radiation.	(171)

Table 3. Characterization of molecular biomarkers of CTCs and their clinical significances in diverse aggressive and metastatic cancers

Cancer type	CTC detection method	Number of CTCs detected by patients/samples	Biomarkers and their diagnostic, prognostic and predictive potentials	Ref.
		Results and clinical significance		
Breast cancer	AdnaTest Breast Cancer Select for detection of EpCAM, MUC1, HER2 and β-actin transcripts	CTCs were detected by AdnaTest in 19% of 502 blood samples from breast cancer patients. Blood samples from 502 primary breast cancer patients *versus* 30 healthy donor samples	29% samples positive for CTCs were characterized by RT-PCR by the expression of at least one EMT marker (twist, Akt2 and PI3Kα) while 97% of 30 healthy donor samples investigated were negative. Moreover, 14% samples positive for CTCs expressed ALDH1 stem cell-like marker while 95% of 30 healthy donor samples were negative.	(38)
Breast cancer	AdnaTest Breast Cancer Select for detection of EpCAM, MUC1 and HER2 transcripts	226 blood samples from 39 metastatic breast cancer patients obtained during their follow-up of chemotherapy, hormonal or trastuzumab antibody-based treatment	CTCs were detected by AdnaTest in 31% of 226 blood samples. The samples positive for CTCs were characterized by RT-PCR by the expression of at least one EMT marker (twist, Akt2 and PI3Kα) and ALDH1 in 62 and 69% cases as compared to 7 and 14% in blood samples negative for CTCs, respectively. The EMT and ALDH1 expression levels in blood samples from patients unresponsive to therapy were of 62 and 44% relative to only 10 and 5% in responders.	(39)
Breast cancer	CellSearch® EpCaM-based immunocapture method	Blood samples from 16 women with metastatic breast cancer with disease progression	Data from analyses performing by CellSearch system and immunostaining have revealed that over 75% of CTCs detected in blood samples from metastatic breast cancer patients co-expressed epithelial markers (EpCAM and E-cadherin) and mesenchymal proteins (vimentin and N-cadherin).	(34)
Breast cancer	Microfluidic capture of CTCs with epithelial- and tumor-specific antibodies directed against EpCAM, EGFR and HER2	Blood samples from 41 patients with metastatic breast cancer at various stages of treatment.	CTCs were detected in 41% of blood samples from breast cancer patients. CTCs expressed epithelial (cytokeratins, EpCAM and cadherin 1) and/or mesenchymal markers (fibronectin, cadherin 2 and serpin peptidase inhibitor, clade E). The enrichment of mesenchymal CTCs was associated with disease progression and expression of TGF-β signaling pathway components.	(189)

| Cancer	Method	Samples	Findings	Ref
Prostate cancer	CellSearch® EpCaM-based immunocapture method	Blood samples from 41 men with metastatic castration-resistant prostate cancer with disease progression	Data from analyses performing by Cell Search System and immunostaining have indicated that over 80% of CTCs detected in blood samples from metastatic CRPC patients co-expressed epithelial markers (EpCAM, cytokeratin and E-cadherin), mesenchymal proteins (vimentin, N-cadherin and O-cadherin) and CD133 stem cell-like marker.	(34)
Ovarian cancer	The monocyte blood fraction containing CTCs was enriched by two-layer density gradient and RNA extracted from the enriched cell fraction	Blood samples from 216 patients with epithelial ovarian cancer obtained before primary treatment and 6 months after adjuvant platinium-based chemotherapy and 39 cases of healthy women	CTCs were detected in 24.5% of the baseline and 20.4% of the follow-up samples. The detection of CTCs correlated with the presence of ascites, sub-optimal debulking and elevated CA-125 and human epididymis protein 4 (HE-4) levels. Moreover, the CTC detection was more frequent in platinum resistant patients than in the group sensitive to platinum treatment and indicative of poor outcome of patients.	(188)
Pancreatic cancer	CellSearch® system	Blood samples from 26 pancreatic cancer patients	CTCs expressing cytokeratins were detected in 42% of blood samples and pancreatic cancer patients with CTCs exhibited significantly shorter overall survival.	(190)
Pancreatic cancer	CellSearch® system or isolation by size of epithelial tumor cells (ISET)	Blood samples from 54 pancreatic cancer patients	CTCs were detected by CellSearch® EpCaM-based immunocapture method and ISET in 40 versus 93% of pancreatic cancer patients, respectively. The immunostaining analyses have also indicated that CTCs expressed variable levels of EpCaM, pan-cytokeratin, E-cadherin, vimentin and cytokeratin 7.	(187)
Colorectal cancer	Pre- and postoperative EpCAM based-flow cytometry analyses	Blood samples from 76 colorectal cancer patients undergoing surgical resection	CTCs were detected in 71% patients preoperatively, and all metastatic patients showed high levels of CTCs. Colorectal tumor surgical resection was associated with a significant decrease in CTCs. A high postoperative level of CTCs was also related to cancer relapse. The progression-free survival rate of colorectal cancer patients without CTCs was increased from 16 to 86%, with a reduction in the risk of disease relapse greater than 90%.	(186)
Lung cancer	Centrifugal force-based separation technique termed dean flow fractionation (DFF)	Blood samples from 20 metastatic lung cancer patients at different stages of treatment versus 20 blood samples from healthy individuals	5-88 CTCs were detected by DFF in all blood samples analyzed from metastatic lung cancer patients while healthy subjects had a negligible number of CTCs. Moreover, the data from immunostaining have revealed that CTCs detected in blood samples from advanced lung cancer patients co-expressed cytokeratin and CD133 stem cell-like marker.	(40)

Non-small cell lung cancer (NSCLC)	CellSearch® system or mutation detection in circulating tumor DNA	Blood samples from 41 patients with relapsed or refractory NSCLC enrolled in a single-arm phase II multicenter trial with erlotinib and pertuzumab	CTCs were detected using CellSearch® system in 78% of NSCLC patients and a greater baseline of CTC counts was associated with a response to treatment by response evaluation criteria in solid tumor (RECIST). A decrease of CTC counts upon treatment of patients was also related with FDG-PET and RECIST response and longer progression-free survival. Data from RT-PCR analyses have also indicated that the detection of mutation in EGFR in ctDNA and DNA isolated from CTCs was associated with a most substantial decrease in CTC counts over the course of treatment of NSCLC patients with erlotinib and pertuzumab.	(185)
Epithelial malignancies	Multimarker quad-μ-nuclear magnetic resonance (μ-NMR)	Biopsies of 58 patients with epithelial malignancies including breast (n=4), gastrointestinal (n=17), genitourinary (n=4), gynecologic (n=7), pancreatic (n=10), lung (n=9) and poorly differentiated adenocarcinoma (n=7) and 6 patients with benign diagnosis	Data from quad μ-NMR expression profiles of the biopsies obtained by the fine needle aspiration of 58 patients with different epithelial malignancies have revealed that the combined analyses of EpCAM, HER2, EGFR and MUC1 markers may be used to achieve the diagnosis of patients with cancer in 99.2% of samples.	(191)
Melanoma	RNA isolation from whole blood samples	Blood sample from 230 melanoma patients, including 154 patients at early stages I-II and 76 patients at late stages III-IV *versus* 152 cases of healthy individuals	Data from quantitative RT-PCR analyses have indicated that 92% of melanoma patients expressed MLANA, TGF-β2, PAX3d, MCAM and ABCG5 multidrug transporter. Importantly, the expression of MLANA and ABCG5 could predict disease recurrence while MLANA expression was associated with a poor outcome of melanoma patients after treatment.	(41)

Figure 1
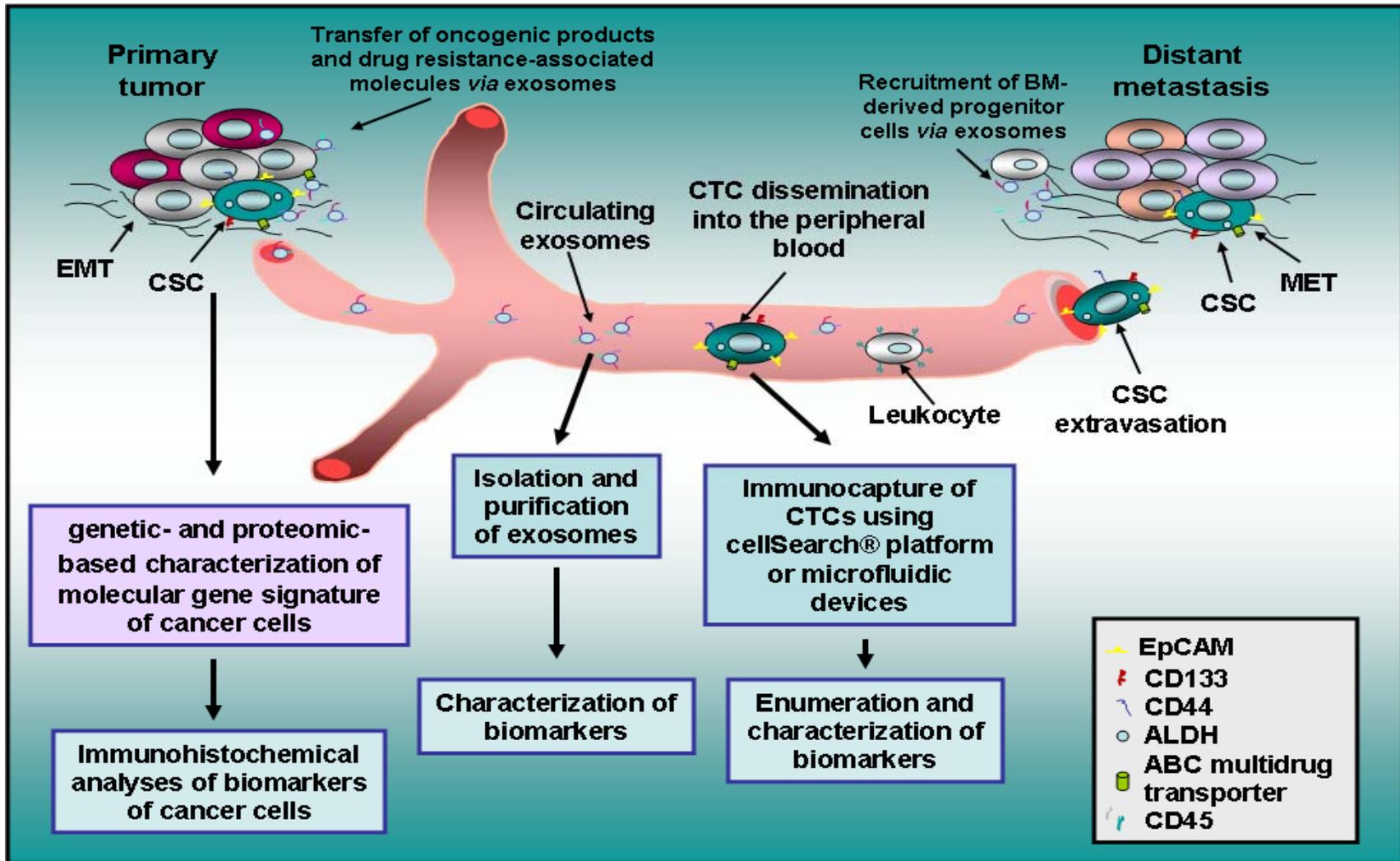
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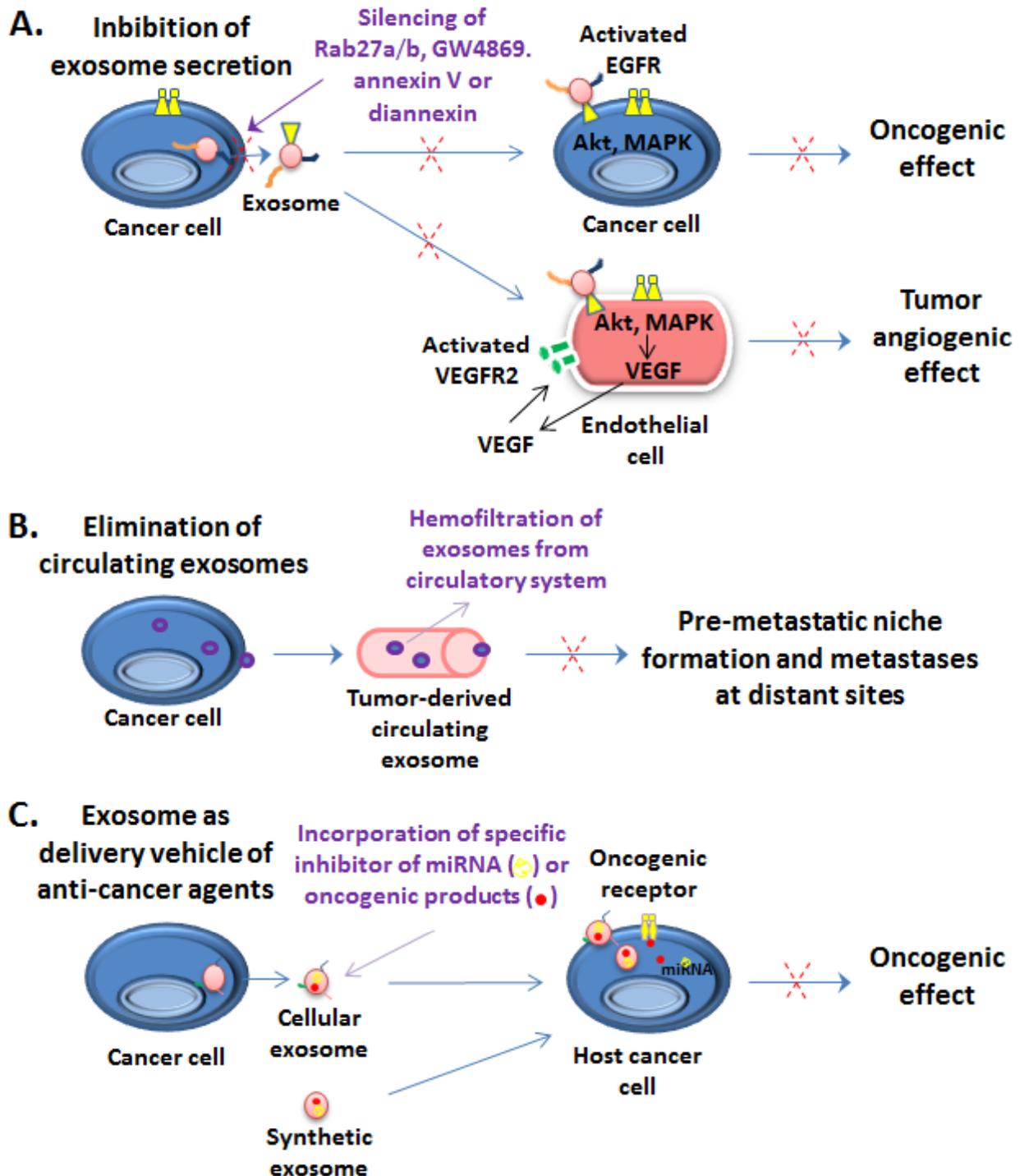
Cancer Epidemiology, Biomarkers & Prevention

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Molecular Biomarkers of Cancer Stem/Progenitor Cells Associated with Progression, Metastases and Treatment Resistance of Aggressive Cancers

Murielle Mimeault and Surinder K. Batra

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