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Detecting Tumor Metastases: The Road to Therapy Starts Here

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Abstract

Metastasis is the complex process by which primary tumor cells migrate and establish secondary tumors in an adjacent or distant location in the body. Early detection of metastatic disease and effective therapeutic options for targeting these detected metastases remain impediments to effectively treating patients with advanced cancers. If metastatic lesions are identified early, patients might maximally benefit from effective early therapeutic interventions. Further, monitoring patients whose primary tumors are effectively treated for potential metastatic disease onset is also highly valuable. Finally, patients with metastatic disease can be monitored for efficacy of specific therapeutic interventions through effective metastatic detection techniques. Thus, being able to detect and visualize metastatic lesions is key and provides potential to greatly improve overall patient outcomes. In order to achieve these objectives, researchers have endeavored to mechanistically define the steps involved in the metastatic process as well as ways to effectively detect metastatic progression. We presently overview various preclinical and clinical in vitro and in vivo assays developed to more efficiently detect tumor metastases, which provides the foundation for developing more effective therapies for this invariably fatal component of the cancerous process.

1. INTRODUCTION

According to the American Cancer Society estimates for the year 2016, 1,685,210 new cases of cancer will be diagnosed while 595,690 people are estimated to die from the disease (American Cancer Society, 2016). With recent advances in therapeutic interventions for treating localized primary tumors, the main cause of death among cancer patients is metastasis. In fact, treating metastatic lesions remains the most challenging obstacle for

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effective therapy in cancer patients (Bacac & Stamenkovic, 2008). Further, early detection of metastatic lesions that might be latent or develop several years after removal of a primary tumor and when a patient is in remission is essential. Thus, developing novel methods for early detection, therapeutic intervention, monitoring, and prevention of metastasis is key to improved patient prognosis. Before we begin our discussion about detecting tumor metastasis, it is important to take a global look at the metastatic process (which is shown schematically in Fig. 1).

The ability of a primary tumor to metastasize to a secondary location in the body is one of the hallmarks of cancer (Hanahan & Weinberg, 2000, 2011). Metastasis from the primary tumor site to a secondary location involves a complex, multistep process (Fisher, 1983; Fisher & Weinstein, 1980; Sahai, 2007). The steps involved in the metastatic process include angiogenesis, epithelial mesenchymal transition (EMT), detachment, degradation of the basement membrane, invasion, migration, intravasation, survival in the circulation, extravasation, and proliferation (Bacac & Stamenkovic, 2008; Fidler, 2003; van Zijl, Krupitza, & Mikulits, 2011). In order for the primary tumor to grow and support its metabolic needs, once it exceeds 1–2 mm in diameter, tumor cells secrete various angiogenic factors to establish a capillary network from the surrounding host tissue, resulting in angiogenesis and tumor vascularization (Fidler, 2003). Epithelial, nonmotile tumor cells transform into mesenchymal, motile cells by the process of EMT, although this step remains controversial (Chui, 2013; Yang & Weinberg, 2008). Primary tumor cells that are transformed or have gained invasive/metastatic abilities must detach and degrade the basement membrane to either move through the basement membrane or between endothelial cells to gain access to the bloodstream/vasculature (Valastyan & Weinberg, 2011).

The tumor cells must then undergo the process of invasion and intravasation to enter the circulation. One of the most common routes for tumor cells to enter into the circulation is thin-walled venules, such as lymphatic channels, which tumor cells can easily penetrate. Tumor cells can migrate to distant locations via the blood and lymphatic circulatory channels. Once in the circulatory or lymphatic system, cancer cells must survive the hostile conditions in the circulation. Tumor cells must resist anoikis (programmed cell death associated with loss of cell–cell contact), they must evade recognition by the host immune system as well as successfully handle other physical stresses of being in the circulation (Joyce & Pollard, 2009). When circulating tumor cells (CTCs) arrest in capillary beds at a distant site, they can exit the circulation via extravasation or proliferate within the vessel. These tumor cells must survive in their new microenvironment, proliferate, and evade detection by the host immune system, as well as establish a blood supply for their growing nutritional needs as well as waste disposal (Hanahan & Weinberg, 2011). The newly formed metastases can create new blood vessels by angiogenesis, co-opt existing blood vessels or grow within an existing blood vessel. The metastatic lesion then needs to grow into a clinically relevant metastatic lesion (Chambers, Groom, & MacDonald, 2002; Fidler, 2002, 2003; Talmadge & Gabrilovich, 2013).

Having briefly overviewed the steps in the metastatic process, we will now discuss the various models used in detecting these metastatic cells. Early detection of metastatic lesions can have a huge impact on overall patient outcomes. Given the importance and urgency of

the need to detect and treat metastasis early, as well as the need to better understand metastasis, several preclinical and clinical models of metastasis have been developed.

2. PRECLINICAL IN VITRO AND IN VIVO MODELS OF METASTASIS

To gain an enhanced, more complete understanding of the process and signaling mechanisms involved in metastasis, researchers have developed several in vitro and in vivo models that have aided preclinical development (Hulkower & Herber, 2011; Jung, 2014).

2.1 In Vitro Models

Several in vitro assays have been used to assess the various steps involved in the metastatic process. As a tumor grows, tumor cells support their growing needs by generating new blood vessels derived as extensions of the existing vasculature through the process of angiogenesis (Chambers et al., 2002). Tumor cells will also use the tumor vasculature to migrate to distant areas. The ability of tumor cells to generate new blood vessels (angiogenesis) can be assessed using the endothelial tube formation assay (Garrido, Riese, Aracil, & Perez-Aranda, 1995). In this assay, endothelial cells are introduced onto extracellular matrix along with conditioned media obtained from the tumor cells. Factors released into the conditioned media by the tumor cells will reprogram the endothelial cells to form tubes which correlate with angiogenic potential. Detailed instructions on performing the endothelial tube formation assay have been published elsewhere (Arnaoutova & Kleinman, 2010).

Cell–cell interactions as well as cell adhesion molecules play a central role in the metastatic process (Bendas & Borsig, 2012). The adhesion assay is utilized to determine changes in the ability of tumor cells to adhere to and interact with different extracellular matrices. During the metastatic process, tumor cells have to adhere to various extracellular matrices to intravasate and extravasate to successfully establish a distant metastasis. Extracellular matrix proteins such as fibronectin, collagen, and laminin are coated onto the bottom of culture dishes and the ability of tumor cells to adhere to the matrix is assessed in the adhesion assay. Several different combinations of extracellular matrix materials precoated onto cell culture plates are also commercially available. The ability of tumor cells to adhere to endothelium can also be assessed using commercially available reagents such as CytoSelect™ Tumor-Endothelium Adhesion assay by Cell Biolabs, Inc. This assay also provides a glimpse at the angiogenic capabilities of tumor cells.

Tumor cells must be capable of migrating from their primary site to a distant organ as well as within the circulation in order to metastasize. The scratch–wound assay is utilized to assess the motility and migration capabilities of tumor cells. Tumor cells are grown in a confluent monolayer. A scratch is made in the confluent layer and the ability of the cells to fill in the scratch or wound is measured over time. Detailed technical instructions on performing scratch–wound assays have been published elsewhere (Cory, 2011; Liang, Park, & Guan, 2007). Another method to determine migratory potential of tumor cells is by using a Transwell or modified Boyden chamber assay. Tumor cells are introduced into the upper chamber and their ability to migrate toward a chemoattractant in the lower chamber is assessed (Chen, 2005).

The ability of tumor cells to invade is another key attribute that ensures the successful development of metastatic lesions. This ability to invade can be assessed using the Boyden chamber invasion assay. The Boyden chamber contains a Transwell membrane with a gel of extracellular matrix proteins. One of the most commonly used Transwell membranes consists of Matrigel. The tumor cells are introduced into the upper chamber and their ability to invade through the Matrigel and toward a chemoattractant in the lower chamber is assessed. Detailed technical instructions on performing Transwell® Invasion assays (Marshall, 2011) and Boyden chamber assays have been published elsewhere (Falasca, Raimondi, & Maffucci, 2011).

Researchers have also developed three-dimensional (3D) culture assays to determine metastatic abilities of tumor cells in a more complex architectural context mimicking conditions in vivo. Here, tumor cells are allowed to grow within a 3D matrix and various attributes of the tumor spheroids that develop within the matrix are assessed (Debnath, Muthuswamy, & Brugge, 2003).

2.2 In Vivo Models

Preclinical in vivo models have been of significant benefit in evaluating innovative methods of detecting tumors and novel therapeutic intervention approaches for potential use in treating human cancers. Several different in vivo models of metastasis have been developed to model human metastatic disease (Khanna & Hunter, 2005; Menezes et al., 2014).

Experimental and spontaneous metastasis mouse models have been developed using both human and mouse cell lines (Price, 2001, 2014). In the experimental metastasis mouse model, human tumor cells with known or suspected metastatic capabilities are injected into immunodeficient mice. The injected tumor cells colonize organs depending on the intrinsic metastatic homing capabilities of the injected tumor cells as well as the site where tumor cells are introduced. Tumor cells that are injected into the tail vein mainly form lung metastases, tumor cells injected into the portal vein colonize the liver, intracardiac injection of tumor cells results in metastasis to a number of organs including bone (Fantozzi & Christofori, 2006), and intratibial injection of tumor cells results in bone metastasis (Park, Kim, McCauley, & Gallick, 2010). For example, when MDA-MB-231 human breast cancer cells are xenografted by injecting the tumor cells into the tail vein, mice develop lung metastasis (Yang, Zhang, & Huang, 2012). Another example is B16 melanoma cells that also form lung metastases when injected via the tail vein (Giavazzi & Decio, 2014). PC-3 prostate cancer cells when delivered via intratibial injection produce osteolytic metastatic lesions while LNCaP prostate cancer cells produce mixed osteoblastic and osteolytic lesions (Park et al., 2010). In these xenograft models, human tumor cells can safely be injected into mice without any rejection because the mice are immunodeficient. However, because these mice are immunodeficient, this model cannot be utilized to study the relevance of an intact immune system in metastatic progression. Other advantages of experimental metastasis models include the ability to control the number of tumor cells introduced into the mouse as well as the ability to target tumor cells to specific sites. Also experimental metastasis models generally develop tumors in a shorter duration of time as compared to spontaneous metastasis models. However, the experimental metastasis models use a more artificial route

of tumor cell delivery to establish metastasis and limited steps within the metastatic process can be assessed using this model (Francia, Cruz-Munoz, Man, Xu, & Kerbel, 2011).

In the spontaneous tumor metastasis mouse model, tumor cells that have or are suspected to have metastatic capabilities are introduced into mice and metastasis is allowed to develop spontaneously (Fidler, 2006). For example, when 4T1 mouse mammary tumor cells are orthotopically injected into the mammary fat pad of syngeneic mice, the cells will spontaneously metastasize to the lungs, liver, bone, and brain (Fantozzi & Christofori, 2006; Yang et al., 2012). When PC-3MM2GL, a highly metastatic variant of PC-3 prostate cancer cells are orthotopically implanted intraprostatic, 100% lymph node metastases develop in 4 weeks (Park et al., 2010). Utilizing syngeneic mouse models are useful when evaluating the role of the immune system and the immune components in metastasis. Other advantages of using spontaneous metastasis models include the ability to follow and determine the mechanisms of metastatic spread via a more natural route that more closely resembles clinical disease as well as the ability to follow all of the steps involved in the metastatic process (Francia et al., 2011). The drawbacks of spontaneous metastasis models include prolonged time required for development of metastases and asynchronous development of metastatic lesions at multiple locations (Francia et al., 2011).

Researchers have also developed genetically engineered or transgenic mouse models that spontaneously develop tumors and metastases (Menezes et al., 2014; Smith & Muller, 2013). In these models, the genetic makeup of the mouse is altered to either inhibit the expression of a tumor suppressor gene or overexpress an oncogene or combinations of the two, so that mice will spontaneously develop tumors and metastases over their normal lifespan, sometimes as rapidly as a couple of months (Eklund, Bry, & Alitalo, 2013; Fantozzi & Christofori, 2006; Husemann & Klein, 2009). In addition, mice that develop tumors in a specific region have been developed by utilizing tissue-specific promoters so that the desired tumor suppressor is suppressed in the specific tissue while the desired oncogene is overexpressed in the specific tissue. These models have an intact immune system that facilitates studying the role of the immune system and immune components as well as the microenvironment in tumor metastasis. For example, the MMTV-PyMT transgenic mouse model has been used to model breast cancer progression and metastasis. In this model, the PyMT (polyoma virus middle T antigen) is expressed under the transcriptional control of the MMTV (mouse mammary tumor virus) promoter, which gives rise to multifocal mammary adenocarcinomas in all the mammary glands of female mice with 100% incidence and metastatic lesions develop in the lungs and lymph nodes by 3 months of age (Guy, Cardiff, & Muller, 1992). Similarly, female MMTV-c-myc transgenic mice that overexpress c-myc under the transcriptional control of the MMTV promoter in the mammary glands develop mammary adenocarcinomas in 5–6 months with a 100% incidence and develop metastatic tumors in the lungs (Stewart, Pattengale, & Leder, 1984). Another example is transgenic mice expressing Kras^{G12D} with Ink4a/Arf deficiency specifically in the pancreas (Aguirre et al., 2003). These mice develop highly invasive and metastatic pancreatic cancers, with all mice succumbing to tumors by 11 weeks. The TRAMP (transgenic adenocarcinoma mouse prostate) mouse model expresses the SV40 T antigen under the transcriptional control of the rat probasin promoter to enhance prostate-specific expression (Parisotto & Metzger, 2013). These mice primarily develop lung and lymph node metastases but occasionally liver,

kidney, and adrenal gland metastases are also observed. Transgenic mice expressing BRaf^{V600E} and silenced for the tumor suppressor Pten develop melanoma with 100% penetrance and metastases develop in the lymph nodes and lungs (Dankort et al., 2009). Although we highlight a small list of interesting transgenic models used to study metastasis, research efforts continue to focus in multiple laboratories to develop genetically engineered mice that accurately recapitulate both primary tumor-specific development and metastases.

Finally, researchers have developed human patient-derived xenograft mouse models that might potentially lead to personalized medicine for cancer patients (Aparicio, Hidalgo, & Kung, 2015; Siolas & Hannon, 2013). In this model, a freshly harvested patient-derived tumor is digested and transplanted into an immunodeficient mouse. To maintain this model, cells are directly passaged from one mouse to another when the tumor burden becomes too high. Patient tumor can be transplanted into the subcutaneous flank of a mouse or orthotopically into the location where the tumor was derived from the patient. For example, human histologically-intact pancreatic cancer specimens have been orthotopically transplanted into athymic mouse pancreas and this model showed extensive local tumor growth, as well as metastases in the liver, regional lymph nodes, adrenal gland, diaphragm, and mediastinal lymph nodes (Fu, Guadagni, & Hoffman, 1992).

3. APPROACHES CURRENTLY USED TO DETECT METASTATIC LESIONS (ON A PRECLINICAL LEVEL)

3.1 Lab Tests/Histopathology

The presence of metastatic lesions in mice with tumors can be detected by sacrificing the mice at the appropriate time point and harvesting the desired organs to be tested for presence of metastases. These organs can be fixed in formalin, paraffin-embedded, sectioned, and placed on glass slides. These slides can then be stained using H&E (hematoxylin and eosin) staining and visually examined for the presence of metastatic cells by a trained pathologist. The slides can also be stained using other techniques to identify the presence of markers of the metastatic process. For example, immunohistochemical analysis (IHC) to identify the presence of CD31 and Factor VIII-related antigen are useful to detect and quantify tumor angiogenesis (Wang et al., 2008). Using human melanoma xenograft mouse model, lectin HPA and adhesion molecules CEACAM-1 (carcinoembryonic antigen-related cell adhesion molecule 1), and L1 expression were assessed by IHC and HPA, CEACAM-1 and L1 were shown to be markers of metastasis (Thies, Mauer, Fodstad, & Schumacher, 2007). Further, metastatic lesions can be probed for expression of protein with known metastatic capabilities to provide additional insight regarding the lesions. For example, melanoma differentiation associated gene-9 (MDA-9/syntenin), ie, frequently over-expressed in metastatic lesions can be assessed to identify metastases and can serve as a biomarker of metastasis (Boukerche et al., 2005).

3.2 Noninvasive Blood Tests

Research is also focused on developing noninvasive methods to detect and monitor tumor metastases using blood tests. miRNAs are small noncoding RNAs that play an important role in tumor progression and metastasis (Baranwal & Alahari, 2010; Zhang, Yang, & Wang,

2014). The presence of different miRNAs has been assessed from blood obtained from mice with tumors. As an example, whole blood was collected from transgenic mice with c-MYC-induced lymphoma, hepatocellular carcinoma, and osteosarcoma, and assessed for the expression of 20–30 miRNAs at different stages of the tumorigenic process (Fan et al., 2008). Specific miRNA expression profiles were identified based on the tumor type and stage. Interestingly, when the tumors regressed the expression of these miRNA returned to normal levels (Fan et al., 2008). Thus, detecting specific changes in miRNA expression from blood can aid in the detection of tumor progression as well as monitoring the efficacy of therapeutic interventions.

A blood-based tumor-activatable microcircle approach was devised for detection of tumors and metastases (Ronald, Chuang, Dragulescu-Andrasi, Hori, & Gambhir, 2015). Nonviral tumor-activatable minicircles encoding human SEAP (secreted embryonic alkaline phosphatase) under the transcriptional control of the tumor-specific survivin promoter along with a transfection agent was introduced systemically via the tail vein of the mouse. The reporter SEAP was only produced in tumor cells and was secreted into the blood stream of the mice. Blood samples collected from mice and plasma were screened for the presence of SEAP, which was indicative of the presence of tumors in the mouse (Ronald et al., 2015).

3.3 Small Animal Imaging

Visualizing metastases in vivo in mice provides an excellent model system to develop and evaluate approaches that may be translatable to visualization of metastases in human patients. The methods discussed earlier detect metastases from ex vivo samples but the methods discussed below detect metastasis in the mouse in vivo allowing for visualization and monitoring of metastasis onset and progression or regression over time without the need to sacrifice the mouse.

In order to directly follow and observe the metastatic process as it evolves over time, researchers have also utilized high-resolution in vivo videomicroscopy in living animals (Chambers, MacDonald, Schmidt, Morris, & Groom, 1998). Using a video camera attached to a light microscope, the movement of cancer cells within the circulation, escape from the circulation and events following escape in the surrounding tissue can be assessed (Chambers et al., 1995; MacDonald & Chambers, 2008).

Bioluminescence imaging (BLI) is a noninvasive, quantitative method of detecting metastases in mice in vivo (Badr, 2014; Contag et al., 1995; Minn et al., 2014). It is generally the first approach to image gene-tagged cells in vivo, when populations of cells rather than single cells are to be studied. Other, more complicated modalities are used for studies beyond small animals, namely clinical translation, as discussed later. Firefly luciferase is the most commonly used luciferase system for BLI. Tumor cells have to be stably transfected to express the firefly luciferase gene (or another detectable agent) and then introduced into the mice to assess experimental or spontaneous metastases. The substrate luciferin is introduced into mice intraperitoneally and metastases are visualized using a bioluminescent imager. Several other luciferase enzymes have been used for BLI including firefly luciferase, Renilla luciferase, Gaussia luciferase, Metridia luciferase, Vargula luciferase, and bacterial luciferase (Close, Xu, Sayler, & Ripp, 2011). Liver metastatic

lesions were successfully visualized by injecting HCT-116 human colon carcinoma cells stably expressing firefly luciferase through the portal vein into the liver of athymic mice (Thalheimer et al., 2013). A slightly different approach is to deliver tumor-specific promoters either singly or within microbubbles via the tail vein of mice in order to detect the presence of tumor metastases. Using the tumor-specific PEG-3 (progression-elevated gene-3) promoter (Su et al., 2005), experimental metastases of human melanoma and breast cancer cells could be visualized in an athymic mouse using BLI (Bhang, Gabrielson, Laterra, Fisher, & Pomper, 2011). This is one instance of BLI being used to study inducible genetic systems, as further highlighted later (Section 3.4).

Magnetic resonance imaging (MRI) is another method for noninvasively detecting metastasis (Gauvain, Garbow, Song, Hirbe, & Weilbaecher, 2005). MRI is one of the standard imaging techniques used in the clinical setting for detection of tumors as well as metastasis (Nakanishi et al., 2007). MRI is performed using a magnetic resonance scanner and a small animal receiver coil. Using different xenograft mouse models of cancer, metastatic lesions in the liver, brain, adrenal glands, and lymph nodes were detected using MRI (Peldschus & Itrich, 2014). Pancreatic tumors and liver metastases could be visualized using MRI in a orthotopic pancreatic cancer mouse model and liver metastasis mouse model (Partecke et al., 2011). Microcomputerized tomography (CT) has also successfully been used to detect metastases in mice. Using a hepatocyte-selective contrast agent and micro-CT, liver metastases established after injecting colon adenocarcinoma cells into the portal vein of mice could be detected (Kim et al., 2008). MR and CT are generally anatomic techniques for detection of metastases in vivo. Radionuclide-based techniques, such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are frequently used for this purpose as well, when higher sensitivity is required at the expense of spatial resolution. Use of radionuclides for preclinical imaging of cancer and metastases has been extensively reviewed (Koba, Jelicks, & Fine, 2013; Vaquero & Kinahan, 2015; Weissleder & Nahrendorf, 2015) and is of course the mainstay of doing so clinically for molecular imaging with [^{18}F]fluorodeoxyglucose, when coupled with CT or MR, and an increasing number of newer, tumor-specific agents (Elsinga & Dierckx, 2014; Spick, Herrmann, & Czernin, 2016). BLI and the radionuclide techniques are also frequently used to detect the presence and appearance of metastases using transcription-induced methods, as discussed later. One example is where MDA-MB-231 breast cancer cells stably expressing HSV1-tk (herpes simplex virus 1 thymidine kinase) was utilized with SPECT to detect bone metastasis in mice (Sanches et al., 2015). The precise location of the bone metastases could be detected by accumulation of the radiolabeled tracer promoted by HSV1-tk using SPECT.

3.4 Molecular-Genetic Imaging (Promoter-Based Protocols)

Molecular-genetic imaging approaches allow visualizing and quantifying biochemical processes at the cellular and molecular level (Bhang & Pomper, 2012; Minn et al., 2014; Pomper & Fisher, 2014). The basic components for molecular-genetic imaging are gene promoters, reporters, and gene delivery vehicles. Several gene promoters that are selectively active in cancer cells have been identified and utilized for molecular-genetic imaging approaches (Minn et al., 2014). Some of these promoters include the PEG-3 promoter (Su, Shi, & Fisher, 1997), the AEG-1 (astrocyte-elevated gene-1) promoter (Bhatnagar et al.,

2014; Kang et al., 2005), the hTERT (human telomerase reverse transcriptase) promoter (Majumdar et al., 2001), a truncated tCCN1 (Cysteine-rich protein 61) promoter (Sarkar et al., 2015), and the survivin promoter (Chen et al., 2004). These promoters that are selectively active in tumor cells can be combined with different reporters and different gene delivery methods in order to visualize tumors and metastatic lesions in vivo. For example, the tumor- and metastasis-specific promoter PEG-3 was combined with the firefly luciferase reporter and HSV1-tk reporter, and delivered using in vivo-jetPEI® (Polyplus transfection) in order to detect micrometastatic disease in mouse models of human melanoma and breast cancer (Bhang et al., 2011) (Fig. 2). Similarly, the tumor-specific AEG-1 promoter was combined with the firefly luciferase reporter and HSV1-tk reporter and delivered using nanoparticles to detect metastases in soft tissues and bone in a mouse model of prostate cancer (Bhatnagar et al., 2014) (Fig. 2).

Use of tumor-specific promoter enables the molecular-genetic imaging applicable to all types of human cancer, whereas the application of conventional target-based imaging is usually limited to certain cancers that express specific targets. In addition, by adopting theranostic reporters, the molecular-genetic approach can provide therapies as well. Systemic injection of the molecular-genetic vector formulated with in vivo delivery nanoparticle is suitable for targeting and imaging metastatic cancers with unknown locations in the body of patients. Although imaging large tumor lesions would be feasible with this technology, detection of micrometastatic lesions may be limited due to relatively lower promoter strength of majority of the tumor-specific promoters (Minn et al., 2014). In order to enhance the expression level of reporters, researchers have developed several molecular biology strategies.

Transcriptional enhancers have been added to expression vectors. Strong enhancers such as human cytomegalovirus (CMV) immediate-early enhancers (Penuelas et al., 2005) and simian virus 40 (SV40) enhancers (Luke et al., 2011) can provide universal elevation of promoter strength. Other enhancers from target- or tissue-specific genes have also been tested. The examples include prostate-specific antigen (PSA) promoter/enhancer combination for targeting prostate cancer (Latham, Searle, Mautner, & James, 2000), prostate stem cell antigen enhancer with uroplakin II promoter for targeting bladder cancers (Wang et al., 2010), and endothelin-1 promoter/enhancer combination targeting tumor neovasculature (Dronadula et al., 2011).

Another successful approach to boost promoter strength was to adopt a research tool originally developed for firefly genetic research, GAL4/UAS system (Brand & Perrimon, 1993). The concept of amplifying transcriptional activity using the two step GAL4/UAS system was first tested by Nettelbeck, Jerome, and Muller (1998). The idea was to create a positive feedback loop of expression by constructing two expression cassettes in the expression vector. First, a weak endothelial cell-specific von Willebrand factor promoter expresses a fusion protein of the DNA-binding domain of Lex A repressor and a strong VP16 activator of HSV1. Second, a reporter gene is under the control of multiple copies of Lex A binding sequences and minimal promoter. The study demonstrated 100-fold increase of tissue-specific expression of the reporter. This useful technique was further developed into a concept of two-step transcriptional amplification (TSTA) for molecular-genetic

imaging (Iyer et al., 2001). Our preliminary in vivo study shows enhancement of sensitivity in detection of small metastatic lesions using the TSTA system (Fig. 3).

Size of the expression plasmid significantly affects the efficiency of transfection. A systemic study demonstrated that the expression level of a reporter was inversely proportional to the size of the expression vector (Yin, Xiang, & Li, 2005). The study also showed that plasmids larger than 5.1 kb exhibited severely decreased transfection efficiency, providing theoretical size limitation for in vivo expression vectors. A recent study using minicircle expression DNA demonstrated 5- to 10-fold enhanced expression of the reporter for cystic fibrosis gene therapy (Munye et al., 2016). This study also showed that the minicircle vector enabled prolonged expression of the reporter and conferred reduced inflammatory response. A practical use of minicircle expression for cancer detection used the survivin promoter upstream of a reporter protein, human SEAP (Ronald et al., 2015). Because SEAP is orthogonal to proteins normally expressed in adult tissue it could be used in a sensitive way to detect cancer in body fluids. Although molecular-genetic imaging was not used in this case, this example shows the versatility of such approaches, namely, that one may merely switch out the promoter, or imaging agent with other detectable substances or even gene-encoded therapeutics.

Promoters other than PEG-3 and survivin (Huang et al., 2011) have been used to good advantage for tumor-selective molecular-genetic imaging and therapy, including hTERT and AEG-1, as alluded to earlier. Because of the modular aspect to the plasmids the imaging reporter and even modality can be switched out. For example, hTERT has been used for tumor-selective expression of the human sodium-iodide symporter (hNIS) for therapy with ^{131}I , a β -particle emitter long used to treat thyroid cancer (Rajecki et al., 2012). In that study the ^{123}I was used as the imaging nuclide in conjunction with SPECT. Delivery of the transgene was through an oncolytic adenovirus. By using hTERT to drive the ferritin heavy chain MR imaging could be used to detect a variety of tumors in vivo, although at lower sensitivity than through methods employing radiotracers (Yang et al., 2016). MR was also the modality of choice for imaging the lysine-rich protein, the expression of which was driven by PEG-3, using chemical exchange saturation transfer (Minn et al., 2015) (Fig. 4). A variety of other tumor-selective promoters, including those that are merely organ-specific, cell-type-specific, and more frankly cancer selective have recently been reviewed (Ahn, 2014; Bhang & Pomper, 2012). Use of promoters that display selective (or enhanced) expression in metastatic cells vs primary tumor cells, mesenchymal tumor cells during EMT, tumor vasculature, cancer stem cells, and hypoxic environments will also be of significant value in designing molecular genetic-based cancer imaging and therapeutic approaches (Talukdar, Emdad, Das, Sarkar, & Fisher, 2016).

3.5 Circulating Tumor Cells

Detecting CTCs in vivo is important and will greatly aid prognostic analysis as well as monitoring efficacy of therapy. Using fluorescently labeled tumor cells, researchers have been able to effectively monitor CTCs in mice (Hoffman, 2014). Using human PC-3 prostate cancer cells, Glinskii and colleagues showed that PC-3 cells growing orthotopically in athymic mice produced more viable CTCs than PC-3 cells growing ectopically

(subcutaneously) (Glinskii et al., 2003). Further, using a dual-color orthotopic coimplantation model of human prostate cancer metastasis in athymic mice, Glinskii and colleagues were able to determine the metastatic potential of CTCs (Glinskii et al., 2003). An equal number of PC-3 GFP-expressing cells isolated from the circulation of mice with orthotopic tumors (CTCs) and parental PC-3 RFP-expressing cells were orthotopically transplanted into athymic mice and interestingly the metastatic lesions were almost exclusively GFP-expressing cells, indicating that cells isolated from the circulation (CTCs) had higher metastatic potential than parental cells (Glinskii et al., 2003). Tumors formed from CTCs isolated from orthotopic PC-3 GFP-expressing tumors in athymic mice could be imaged using the FluorVivo imaging system by INDEC Biosystems and the CTCs could be isolated using immunomagnetic beads coated with anti-EpCAM (epithelial cell adhesion molecule) and anti-PSMA (prostate-specific membrane antigen) within minutes (Kolostova, Pinterova, Hoffman, & Bobek, 2011).

4. APPROACHES USED TO DETECT METASTATIC LESIONS (ON A CLINICAL LEVEL)

In the preceding section, we discussed several approaches for detecting metastatic lesions on a preclinical level. Any diagnostic procedure is considered as perfect only when it displays 100% sensitivity and specificity (ie, everyone with cancer would have a positive test, while everyone without cancer would exhibit a negative test) (Mordente, Meucci, Martorana, & Silvestrini, 2015). Regrettably, not all approaches meet these criteria and have not successfully translated from “bench to bedside.” Here, we provide a general overview of the different “conventional” and “state-of-the-art” processes that are routinely used for identifying metastatic lesions in different cancers.

4.1 Biomarkers for Metastasis

“Biomarker” is defined by US Food and Drug Administration (FDA) as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Taube, Jacobson, & Lively, 2005) that can be detected in circulation (whole-blood, serum, and plasma), secretions (stool, urine, and sputum), or organ biopsies (Kulasingam & Diamandis, 2008; Mordente et al., 2015). Until recently, FDA approved 19 protein-based biomarkers (Mordente et al., 2015). Clinically, although these biomarkers are routinely used for monitoring tumor progression, staging, and in some contexts screening purposes, their correlation with tumor metastasis are not fully reliable and often need confirmation using a second approach, eg, imaging.

PSA is a well-described biomarker for prostate cancer and its utility as a diagnostic tool is established (Wilt, Scardino, Carlsson, & Basch, 2014). In 1994, Vijayakumar et al. retrospectively evaluated 90 patients with prostate cancer, on the basis of initial serum-PSA level and bone scans, and found that patients with PSA more than 10 ng/mL had evidence of bone metastasis (Vijayakumar, Vijayakumar, Quadri, & Blend, 1994). Similar results were obtained in another retrospective study conducted by Kamaleshwaran et al. where the cut-off value for PSA was set at 20 ng/mL or greater for predicting bone metastases

(Kamaleshwaran et al., 2012). In subsequent studies, to determine the correlation between PSA levels and bone metastasis risk, Moreira et al. (2015) developed a bone metastasis predictive table using serum PSA level that was further validated by Freedland et al. (2016). According to these two recent studies the cut-off value for PSA was 5 ng/mL and below that level the incidence of skeletal metastasis was very rare (Freedland et al. (2016). In another pooled study using 8644 patients (from 23 studies), the investigators observed that the likelihood of a positive bone scan increases markedly in patients who exhibit a PSA level 20 ng/mL, locally advanced disease, or a Gleason score 8 (Abuzallouf, Dayes, & Lukka, 2004; Briganti et al., 2014). Overall, all of these retrospective studies argue that PSA levels are a valid predictor of bone metastasis, however, which cut-off value most accurately predicts risk is unclear.

Mucins are a family of high-molecular weight glycoproteins expressed/produced by epithelial cells (Nicolini, Ferrari, & Rossi, 2015). Members of this family are involved in breast cancer development and altered expression is associated with cancer progression (Nicolini et al., 2015). Carbohydrate antigen 15.3 (CA15.3) is the most common member in this family and approved by FDA as a biomarker for breast cancer and some other malignancies such as ovarian cancer, endometrial carcinoma, and nonsmall-cell lung cancer (Molina et al., 2008; Moore et al., 2008; Nicolini et al., 2015). Geraghty et al. demonstrated that the serum CA15.3 level was elevated in 50–80% of breast cancer patients with metastasis (Geraghty, Coveney, Sherry, O'Higgins, & Duffy, 1992). In a study conducted in 2007, Keshaviah et al. investigated the relevance of this biomarker with development of recurrence and found that the risk of recurrence increased by 30% in breast cancer patients with abnormal levels of CA15.3 (Keshaviah et al., 2007). In another study, 88% (23 out of 33) patients showed a positive correlation between serum CA15.3 levels and breast cancer bone metastasis (O'Brien et al., 1992). In a prospective study (Kokko, Holli, & Hakama, 2002), 243 patients with localized disease were followed for relapse and metastasis development after primary treatment. Fifty-nine relapses were noticed within 5 years and 36% of these had an elevated level of CA15.3. More than 50% of bone metastasis in these subjects demonstrated a higher level of CA15.3, supporting the use of CA15.3 as an alternative to conventional bone scintigraphy (Byrne, Horgan, England, Callaghan, & Given, 1992). Begi et al. compared bone scintigraphy with serum CA15.3 levels and found a weak correlation between the number of metastases and CA15.3 levels (Begi et al., 2005). However, a significant difference was observed in CA15.3 levels when comparing patients with metastases to patients without metastases. This particular study also compared CEA (carcinoembryonic antigen, another serum biomarker approved by the FDA) levels with bone metastasis development and observed a positive correlation between CEA levels and the number of metastatic lesions (Begi et al., 2006). CA19.9, another member of the mucin family is being considered as a potent diagnostic factor for pancreatic cancer with overall sensitivity of 81% and specificity of 90% (Duffy et al., 2010), although the utility of this factor to screen pancreatic cancer is questionable. Kim et al. analyzed the serum levels of CA19.9 among 84 pancreatic cancer patients who had undergone curative resection and found a positive association between CA19.9 levels in about 69% of the patients that developed distant metastasis within 6 months (Kim et al., 2011). Additionally, this study

confirmed that patients with higher preoperative levels of CA19.9 also had a higher tendency to develop distant metastasis (Kim et al., 2011).

Epidermal growth factor receptor (EGFR) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER2-neu) are two additional bio-markers detected in tissue specimens and the correlation of these biomarkers with disease progression are well studied although their diagnostic value in metastasis remain to be confirmed. In a recent study, Wang and Wang systematically performed a meta-analysis to define EGFR mutations in primary and matching metastatic nonsmall cell lung cancer (NSCLC) and found that EGFR mutations are present both in primary and metastatic NSCLC lesion, and therefore routine analysis of EGFR is not recommended in primary and metastatic tumors (Wang & Wang, 2015). In another study, Westood and colleagues analyzed 12 databases and verified EGFR mutation status in NSCLCs (Westwood et al., 2014). Consistent with Wang and Wang's (2015) observation they also did not find any greater accuracy for EGFR mutation as a diagnostic measure. However, some positive correlations of EGFR with breast cancer metastasis are evident. Gaedcke et al. demonstrated that EGFR expression was increased by 40% in brain metastases compared to primary tumors, which showed only 16% EGFR expression (Gaedcke et al., 2007). However, it is important to also note that 75–85% of primary and brain metastatic tumors were shown to have constant EGFR expression (Gaedcke et al., 2007; Grupka, Lear-Kaul, Kleinschmidt-DeMasters, & Singh, 2004). In agreement with breast cancer data, Deng et al. analyzed and concluded that higher expression of EGFR in metastatic lymph nodes may be more accurate in predicting survival than in primary or metastatic tissues (Deng et al., 2009).

A study of elevated HER2 levels, a protein-based biomarker, detected in tissue specimens of primary tumors and axillary lymph node or distant metastases has been shown to correlate in several clinical studies (Brufsky et al., 2011; Kuba et al., 2014; Shao et al., 2011). In contrast, multiple studies indicate that HER2-positive metastases with negative primary tumors are more frequent (Dieci et al., 2013; Jensen, Knoop, Ewertz, & Laenkholm, 2012; Strien, Leidenius, von Smitten, & Heikkila, 2010; Xiao, Gong, Han, Gonzalez-Angulo, & Sneige, 2011). Thus, Rossi et al. concluded that this phenomenon could be correlated with enhanced tumor aggressiveness or with an underestimation of HER2 protein overexpression in the primary tumor by the pathologist (Rossi et al., 2012). Although several protein-based biomarkers have been used to detect or predict the propensity of metastatic lesion development, until now clinical oncologists rely heavily on various imaging modalities for higher accuracy and sensitivity.

4.2 Imaging Procedures

X-ray, radiographs, computed tomography scan (CT scan), nuclear imaging including PET and SPECT, MRI are few examples of currently available approaches in the clinical arena (Minn et al., 2014; Pomper & Fisher, 2014). In this particular section, we will focus on the clinical applications of these approaches, particularly in the context of bone metastasis, which is very difficult to detect using conventional protein-based biomarkers.

Bone is the third most common site for cancer metastasis and a major reason for mortality for prostate and breast cancer (Bussard, Gay, & Mastro, 2008; Yu, Tsai, & Hoffe, 2012).

Vertebrae, pelvis, ribs, and the ends of long bones are preferred destinations, whereas mandible, patella, and distal extremities are less common for metastases (Bussard et al., 2008; Roberts et al., 2010; Saha, Burke, Desai, Vijayanathan, & Gnanasegaran, 2013). Detection of bone lesions or metastasis are always challenging in comparison with lesions in soft tissue or solid organs, such as the lungs or liver. Skeletal scintigraphy (SS), radiography, PET, and MRI are the most common approaches that are currently being used to detect bone metastases. Plain radiographs are recommended to assess the risk of pathological fracture; however, this approach is not of adequate sensitivity to routinely screen for asymptomatic metastasis (Costelloe et al., 2009; Roberts et al., 2010). Additionally, this approach is not suitable for monitoring treatment response (Bussard et al., 2008; Vassiliou et al., 2011). Computed tomography (CT) has advantages over radiography based on resolution, and sensitivity/specificity (O'Sullivan, Carty, & Cronin, 2015), and evaluating treatment responses (Vassiliou et al., 2011). It is an excellent approach to detect bone metastasis in bone marrow before initiation of bone destruction—thus pertinent to early diagnosis (Bauerle & Semmler, 2009). PET is another imaging modality that can also detect skeletal metastasis and it is superior in terms of spatial resolution (O'Sullivan et al., 2015). The sensitivity of PET is dependent on the type of radiotracers employed. ^{18}F -FDG and ^{18}F Sodium fluoride (NaF) are two common radiopharmaceuticals most frequently employed to detect skeletal metastasis. Existing literature suggests that ^{18}F NaF-PET is both sensitive and specific with improved resolution and better discrimination capability to distinct normal and abnormal bone (Langsteger, Heinisch, & Fogelman, 2006). Other tracers, such as ^{18}F -choline, ^{11}C -choline (half-life of 20 min) are also used in staging bone disease in prostate cancer. ^{11}C -choline PET may have other advantages over ^{18}F -FDG PET for detection of pelvic disease and bone metastases (Messiou, Cook, & deSouza, 2009). PET is a functional rather than anatomic imaging approach such as CT and depends on the uptake of radiotracers. In patients with primary osteosclerotic metastasis from prostate cancer, ^{18}F -FDG PET has shown less sensitivity due to potential uptake problems. The other disadvantage of PET is inability to assess the treatment response in patients who underwent hormone therapy, a phenomenon known as “flare phenomenon” characterized by enhanced uptake of radionucleotides resulting in false-positive findings (Lecouvet et al., 2014; Vassiliou et al., 2011). In breast cancer patients, PET scans are not recommended in some contexts (Khan et al., 2007). In a retrospective study conducted in University of Kansas only 2% of patients were confirmed as having metastasis although 18% were primarily diagnosed with cancer by PET scanning (Khan et al., 2007). However, it is more reliable to determine locally advanced breast cancer and to detect extra-axial nodal disease (Bellon et al., 2004; Fuster et al., 2008; Mahner et al., 2008). Therefore, this approach is optional to detect breast cancer metastasis.

As per guidelines established by the National Comprehensive Cancer Network, MRI is the first line imaging modality for cancer of the head and neck, central nervous system, prostate, and hepatobiliary system (Spick et al., 2016). With respect to sensitivity and specificity issues, MRI is comparatively better than PET in detecting metastasis spreading in the marrow cavity and extension of tumors toward surrounding tissues (Costelloe et al., 2009; O'Sullivan et al., 2015). It is extremely beneficial to detect early tumor cells seeding into the hematopoietic compartment (Tombal & Lecouvet, 2012). In one study, MRI was able to

detect over 37.5% of positive cases, which were primarily considered as indecisive by other approaches such as bone scan and plain X-ray (Lecouvet et al., 2007; Messiou et al., 2009). The development of whole-body MRI advances our capabilities to survey the entire body for detecting any marrow abnormalities, which was further improved using perfusion (DCE) and diffusion-weighted imaging (DWI) that refine the assessment of lesions during the (early) phases of therapy, providing tools to evaluate the efficacy of treatments targeting bone lesions (Attariwala & Picker, 2013; Dutoit, Vanderkerken, & Verstraete, 2013; Essig et al., 2013). It should be noted that DWI approach is extremely valuable to detect metastasis in ribs, which are very difficult through conventional MRI (Lecouvet et al., 2010; Venkitaraman et al., 2009). A meta-analysis was conducted by Yang and colleagues to compare the different approaches (eg, ^{18}F FDG PET, CT, MRI, and bone scintigraphy) used for detection of bone metastasis in clinical settings and concluded that PET and MRI are equivalent and both significantly more accurate than bone scan and CT (Yang, Liu, Wang, Xu, & Deng, 2011) to detect bone metastasis. This conclusion is based on data from 67 studies published during 1995 to January 2010 in MEDLINE and EMBASE database.

Diagnostic accuracy can be significantly enhanced by combining two approaches, introduced as “hybrid technology” in the field of cancer imaging (Cherry, 2009; Yoo, Lee, & Lee, 2015). PET/CT, an example of this hybrid technology which utilizes the metabolic information of PET with the anatomic detail of CT, which overcomes the inherent barriers of the individual approach. In a further refinement of the combined approach, PET/MRI was recently introduced with excellent soft tissue resolution (Partovi et al., 2014). The superiority of PET/MRI over PET/CT in cancer diagnosis were confirmed in multiple recent studies including oncolytic bone lesions (Beiderwellen et al., 2014), liver metastasis (Beiderwellen, Geraldo, et al., 2015), detecting malignant/benign lesions in recurrent breast cancer patients (Sawicki et al., 2016), recurrent female malignancies such as ovarian cancer (Beiderwellen, Grueneisen, et al., 2015), thyroid cancer (Nagarajah et al., 2011), pancreatic (Nagamachi et al., 2013), and head and neck (Queiroz & Huellner, 2015) cancers. In pancreatic and head and neck cancer studies, although PET/MRI is sensitive over PET/CT, the differences were not statistically significant. A very recent review compared these two approaches in different malignancies and concluded that both function equally well for cancer assessment (Spick et al., 2016). PET/MRI has advantages over PET/CT in clinical management, detecting bone metastasis and locating intraprostatic sites of disease (Spick et al., 2016). On the other hand, PET/CT approach is advantageous in detecting pulmonary metastasis in some contexts (Spick et al., 2016). However, it is too early to reach a firm conclusion due to the limited numbers of comparative studies.

Despite technical advantages of imaging modalities, clinical oncologists often face several obstacles in bone metastasis detection that includes age-associated benign pathologies that might mimic the signal from metastatic cells, “flare phenomenon” (increased uptake of radiotracer) after hormone therapy, and the difficulty in detecting individual lesions when they are closely spaced (termed as superscan pattern frequently observed with elderly patients, late stage of disease, and individuals who are predisposed to bone metabolic disorders) (Lecouvet et al., 2014). Other issues such as scan duration ($^{99\text{m}}\text{Tc}$ -bisphosphonates must be imaged for several hours, whereas ^{18}F -NaF by 10 min) (Win & Aparici, 2014), longer on-camera acquisition, soft tissue uptake, extraosseous uptake of ^{18}F -

NaF due to hypocalcemia, calcified soft tissues might also impose some technical challenges to accurately detect the lesions (Lecouvet et al., 2014).

4.3 Circulating Tumor Cells

Presence of circulating tumor cells in blood was first reported almost 140 years ago and, very recently CTC research expanded rapidly to emphasize its potential as both a diagnostic and prognostic marker of cancer. As of March 2016, there were approximately 17,571 publications and more than 767 clinical trials under the search term “circulating tumor cells” in PubMed/Clinicaltrial.gov database reflecting the logarithmic expansion of this emerging technology. Extremely low numbers of CTCs in the circulation (as low as 1 CTC in 10^6 – 10^7 leukocytes of peripheral blood; Hong & Zu, 2013), lack of universal CTC detection antibodies, absence of appropriate sensitive methods for rapid molecular characterization of CTCs, and the need for sensitive technical equipment have proven to be challenges in adapting this approach for routine screening (Hong & Zu, 2013). Additionally, various methodologies practiced by different laboratories, lack of reference samples, selection biases, use of diverse capture antibodies from different sources, and oversimplification of cytopathology processes significantly impact on the validity of outcomes (Hong & Zu, 2013). Despite these limitations, a substantial number of clinical studies have shown the power of the CTC technology in patients with multiple cancer indications. The seminal work from Cristofanilli and colleagues revolutionized the clinical applications of CTCs and established the correlation of CTCs with progression-free survival and overall survival in patients with metastatic breast cancer (Cristofanilli et al., 2004). Following this study, a plethora of supportive studies have been published by multiple independent laboratories (Giordano & Cristofanilli, 2012; Giordano et al., 2013; Lianidou, Strati, & Markou, 2014; Pierga et al., 2012). In addition to tumor progression, the CTC approach has also been successfully applied to follow treatment responses including adjuvant chemotherapy in a phase II trial, after surgery (Ignatiadis et al., 2007; Pierga et al., 2008). The clinical relevance of CTCs has also been studied in prostate cancer at both early and advanced stages. In a pilot study, Lowes et al. demonstrated that CTCs can be detected at early stages of prostate cancer and may be pertinent to follow therapeutic responses (Lowes et al., 2012). Castration resistant prostate cancer (CRPC) is an advanced stage disease and both localized (Helo et al., 2009) and metastatic stages (Danila et al., 2007; de Bono et al., 2008; Scher et al., 2009) of CRPC correlated with CTCs in these independent studies. Interesting phenomenon was observed by Armstrong et al. (2011) that CTCs isolated from patients expressed both mesenchymal and epithelial markers, and the numbers of CTCs served as surrogate marker. Molecular characterization of CTCs isolated from melanomas showed some inconsistencies when BRAF mutation (Sakaizawa et al., 2012), a signature for 81% cases of melanoma status (Kitago et al., 2009), was analyzed although the clinical significance of CTCs in patient’s blood was correlated with disease-free survival and overall survival (Hoshimoto et al., 2012). Presence of CTCs in pancreatic cancers correlated with an unfavorable prognosis (Tjensvoll, Nordgard, & Smaaland, 2014). In 2013, Han et al. published a meta study with total 623 pancreatic cancer patients and established a positive correlation with CTCs and disease outcome (Han, Chen, & Zhao, 2014). This particular study also compared the survival among CTC-positive and CTC-negative patients, and the overall survival was worse in the latter group further supporting the prognostic potential of

CTCs in the context of pancreatic cancer (Han et al., 2014). The prognostic value of CTCs in the contexts of resectable colorectal liver metastasis and metastatic colorectal cancer were studied in 11 independent publications. As per this summary report (Huang et al., 2015), liver metastasis are more prominent in CTC-positive patients and the presence of reduced CTCs are associated with overall progression-free survival. The study also suggested that the presence of CTCs could act as an indicator for treatment response (Huang et al., 2015). Apart from these studies, promising correlations of the presence of CTCs with head and neck (Wu et al., 2016), bladder cancers (Gazzaniga et al., 2014) were also reported by different groups. Although lung cancer mortality is a major clinical concern, however, the correlation of CTCs with lung cancer detection was less predictive at least with current sets of methods, which mostly rely on use of epithelial marker to identify CTCs. In lung cancer, CTCs often exhibit non-epithelial characteristics (Wu et al., 2015; Zhang, Ramnath, & Nagrath, 2015). In this context, developing appropriate reagents to detect both epithelial and mesenchymal markers will be beneficial. Despite this challenge, attempts were taken and positive correlations between CTCs with lung cancer were reported (Lecharpentier et al., 2011; Zhang et al., 2015; Zhu et al., 2014). Regarding treatment response, a meta-analysis considering 12 relevant studies demonstrated that prior to treatment CTCs correlated with lymph node status, distant metastasis, and disease staging, however, post-treatment CTCs only correlated with staging (Ma et al., 2012). Including the earlier mentioned meta-analysis, a number of meta-analyses were conducted in different cancers such as gastric cancer (Wang, Wei, Zou, Qian, & Liu, 2016), metastatic breast cancer (Lv et al., 2016), ovarian cancer (Cui, Kwong, & Wang, 2015; Zhou et al., 2015), head and neck (Wang, Cui, Xue, Tong, & Li, 2015), colorectal cancer (Huang et al., 2015), liver cancer (Jin, Peng, & Wu, 2013), prostate cancer (Wang et al., 2011), bladder and urothelial cancer (Wang et al., 2011), and positive correlations with CTCs were established.

Sample volume and sampling number are the major determining factors in quantifying CTCs. In current settings, a small amount of blood (around 7.5 mL, 0.15% of our total volume of blood) is used to detect CTCs. It can be argued that this amount of sample may not be statistically adequate to represent the whole circulation. A study compared the average CTCs in 7.5 vs 30 mL from 15 patients and the results suggested that larger volume is preferable for detecting CTCs (Lalmahomed et al., 2010). As per Allan and Keeney's mathematical model, at least 20 mL of whole blood sample needs to be analyzed to detect the lower frequency (1 CTC in 10^7 leukocytes) (Allan & Keeney, 2010). Multistep and complex sample processing represent other issues that need to be optimized to circumvent inconsistency. Additionally, operator variability and data interpretation could also impact the results of CTCs in a clinical setting (Hong & Zu, 2013). Regardless of the primary success of this approach that has been reported in various studies, monitoring CTCs is not well accepted as a diagnostic tool by different oncology associations such as the American Society of Clinical Oncology, the National Academy of Clinical Biochemistry (reviewed by Hong & Zu, 2013; Sturgeon et al., 2008; Zhang et al., 2015). Turnaround time for typical *Point of Care* (normally within 1 h, but 30 min is preferred) (Kilgore, Steindel, & Smith, 1998; Louie, Tang, Shelby, & Kost, 2000) is another limitation for practical implication of CTC approaches (Hong & Zu, 2013). However, considering all the published results from several clinical studies CTC biology might have significant future potential in the clinical

arena. At this stage, major advances are needed to improve the tools and methodologies for monitoring CTCs, which if achieved might significantly enhance the power and accuracy of this approach for monitoring patient cancers noninvasively.

5. METASTASIS DETECTION/THERAPY: COMBINING IMAGING WITH THERAPY (THERANOSTICS)

“Theranostics” integrates the processes of diagnosis and therapy (Baum & Kulkarni, 2012), and represents a rapidly emerging area in cancer. For efficient disease management, being able to diagnose, target, and monitor therapeutic responses are essential. In the clinical arena over the past few decades’, significant achievements have been made individually in these three aspects of patient care. The “theranostic” concept has merged these three independent aims into a single platform and examples of successful applications have been documented in preclinical animal and in limited clinical studies establishing this approach as a promising therapeutic strategy.

Theranostic approaches are relatively new and studies are currently conducted to provide evidences for developing effective treatment regimes in cancer biology including gene therapy, chemotherapy, and radiation therapy. Gene therapy is an exciting area in cancer research with additional applications in other inherited genetic diseases such as cystic fibrosis where a faulty gene is being replaced with a correct gene (Das et al., 2015). Delivery of a “gene of interest” specifically in targeted cells is always challenging. Various approaches including both viral- and lipid-based molecules are being optimized for efficient delivery of nucleotides (Das et al., 2015). Recently, strategies have been developed where both vehicles and nucleotides are labeled with organic dyes, which can be tracked by the imaging techniques (Hong, Yang, Zhang, & Cai, 2010). Quantum dots are an alternative to organic dyes and have advantages over fluorescent dyes for nucleic acid trafficking (Walling, Novak, & Shepard, 2009). However, these latter approaches are still predominantly at an experimental stage in the laboratory and no information is available relating to the successful application of these strategies in the clinic.

Microbubbles, gas filled spheres currently used as FDA-approved ultrasound contrast agents (Castle et al., 2013), have been evaluated as drug and therapeutic virus carriers in numerous studies and have potential as theranostic tools (Kiessling, Fokong, Koczera, Lederle, & Lammers, 2012). Application of ultrasound in conjunction with microbubbles can enhance the acoustic forces that facilitate drug/therapeutic delivery (Azab et al., 2012; Dash et al., 2011; Sarkar et al., 2015) across various biological barriers including the blood–brain barrier (Meairs, 2015). Although it is beyond the scope of this review, but it is worth noting that utility of microbubbles as a therapeutic in thrombolysis in addition to imaging has been validated clinically (Molina et al., 2006). Theranostic property of microbubbles was initially observed by Leong-Poi et al. in a study where microbubbles containing a VEGF expression plasmid (permitting VEGF expression) were injected into the rat to treat arteriogenesis (Leong-Poi et al., 2007). In the same year, Rapoport and colleagues provided more confirmative evidence from in vivo studies showing the success of this approach (Rapoport, Gao, & Kennedy, 2007). Doxorubicin-loaded nanobubbles were injected which extravasated

into the tumor and coalesced into microbubbles at physiologic temperatures (Rapoport et al., 2007). Ultrasound was applied to visualize the tumor site as well as trigger the destruction and delivery of drugs that eventually killed the tumor. Recently, research has focused on developing target-specific microbubbles by decorating their surface, which can enhance and expand the utility of this approach in the clinic.

Nanoparticles, which are commonly used to deliver drug(s) (Mudshinge, Deore, Patil, & Bhalgat, 2011), also have proven amenable as imaging and therapeutic modalities (Kievit & Zhang, 2011). Indeed, although nanoparticles as theranostics are still in their early stage of development, a substantial number of studies support their potential (Xie, Lee, & Chen, 2010). Iron oxide nanoparticles (IONPs), also known as magnetic nanoparticles due to their magnetic properties are used as contrast agents in MRI (Bu et al., 2012). For example, several dextran-based IONPs are approved and currently used for detection of liver and spleen lesions by AMAG pharmaceuticals (Xie et al., 2010). Various research groups verified IONPs as a carrier for chemotherapeutics (eg, methotrexate, paclitaxel, doxorubicin) and nucleotides (Hwu et al., 2009; Xie et al., 2010). Besides their roles as contrast agents and therapeutic carriers, their magnetic properties have also been utilized for therapy of tumors (Wadajkar et al., 2013). Application of external alternating magnetic fields can convert electromagnetic energy into heat, which raises the temperature of the tumor above 43°C resulting in thermal killing of tumor cells (Shen et al., 2015). Gold nanoparticles, due to stability, biosafety, and ability to be modified for better delivery and targeting are routinely used to deliver a wide variety of therapeutics (Khan, Rashid, Murtaza, & Zahra, 2014). Utility of gold nanoparticles as imaging or diagnostic agents is related to particle absorption spectrum and upon application of laser irradiation, gold nanoparticles serve as energy transducers and induce photothermal killing as shown by different studies including breast (Au et al., 2008; Jin, Hong, Kakar, & Kang, 2008), glioblastoma (Jin et al., 2008), oral (El-Sayed, Huang, & El-Sayed, 2006), and urothelial cancer (Chen, Wu, & Chen, 2015). Other nanoparticles such as carbon nanotubes, silica-based nanoparticles have also demonstrated theranostic properties in specific situations (Xie et al., 2010).

Molecular-genetic approach is suitable for theranostics of metastatic tumors. One can take advantage of a single reporter that can serve as both an imaging and therapeutic gene. One of the most widely utilized theranostic genes is HSV1-tk. Many HSV1-tk-specific substrates have been developed for imaging and therapeutic applications and these include pyrimidine nucleoside derivatives and acycloguanosine derivatives. 2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil (FIAU), 2'-fluoro-2'-deoxy-5-methyl-1-β-D-arabinofuranosyl-5-iodouracil (FMAU), and 2'-fluoro-2'-deoxy-5-ethyl-1-β-D-arabinofuranosyl-5-iodouracil (FEAU) belongs to the pyrimidine nucleoside derivatives. Acyclovir, ganciclovir, penciclovir, and 9-(4-fluoro-3-hydroxymethylbutyl)guanine (FHBG). FIAU, FMAU, FEAU, and FHBG labeled with ¹⁸F can serve as PET tracers. FIAU labeled with a therapeutic radioisotope such as ¹³¹I can be utilized as radiopharmaceutical therapy (Yaghoubi et al., 2005). Finally, acyclovir, gan-ciclovir, and penciclovir are potent prodrugs that require HSV1-tk to be converted to active ingredients. Clinical application for theranostic applications using HSV1-tk have been tested in human glioma (Jacobs et al., 2001), liver cancer (Penuelas et al., 2005), and relapsed allogenic stem cell transplant patients (Eissenberg et al., 2015). Somatosatin respect 2 (SSTr2) has been tested as a

theranostic gene. Radiolabeled octreotides such as [^{123}I]Tyr3-octreotide (Krenning et al., 1989), [^{111}In]DTPA-D-Phe-octreotide (Bakker et al., 1991), and [$^{94\text{m}}\text{Tc}$]Tyr3-octreotate (Rogers et al., 2005) have been tested for nuclear imaging probes targeting SSTR2. Y-90 DOTA-Phe1-Tyr3-octreotide has been used to treat neuroendocrine tumors (Bushnell et al., 2004). NIS can also be used as a theranostic gene by using [$^{99\text{m}}\text{Tc}$]pertechnetate and [^{124}I]NaI as SPECT and PET tracer, respectively (Chung, 2002), and [^{131}I]NaI (Dadachova & Carrasco, 2004) or [^{188}Re] (Kang et al., 2004) for therapeutic radionuclides.

6. CHALLENGES FACED IN DETECTING METASTATIC CELLS

Even with our ever-increasing understanding of the mechanism(s) underlying cancer progression and metastatic spread, detecting metastatic cells still remains challenging. Detecting metastases at their inception would greatly aid in overall patient outcome as targeted therapies could be administered at the onset of this progressive process. Further inhibiting the growth of metastases at a distant location will greatly benefit overall patient outcomes. Although researchers have determined multiple molecular changes, immunologic, and genetic factors that support the metastatic process, as well as several methods that aid in detection of metastases, these findings have not been easy to translate into the clinical setting. Conventional methods of detecting metastatic lesions such as MRI and CT are not sensitive enough to detect micrometastases.

CTCs can play a crucial role in the development of metastasis. Several groups have explored techniques to detect CTCs (Hong & Zu, 2013; Lurje, Schiesser, Claudius, & Schneider, 2010). However, CTCs are present at extremely low concentrations—as low as one CTC in 10^6 – 10^7 leukocytes in peripheral blood of cancer patients (Hong & Zu, 2013). Several devices/instruments have been developed to detect CTCs, including the only US FDA-cleared CellSearch[®] system by Veridex which captures and enumerates CTCs in cancer patients with metastatic breast, prostate, and colorectal cancer (Andree, van Dalum, & Terstappen, 2016). However, challenges in detecting CTCs still remain and have been described in detail elsewhere (Andree et al., 2016; Hong & Zu, 2013; Pantel & Alix-Panabieres, 2010).

7. CONCLUSIONS AND FUTURE DIRECTIONS

As discussed earlier, several in vitro and in vivo preclinical and clinical methods have been developed to gain a better understanding of the metastatic process. However, while several research groups have attempted to dissect the various steps of metastasis as well as develop methods to detect metastatic lesions; it is clear that several challenges still remain and a lot still needs to be done before these techniques become commonplace in the clinic for detecting metastatic lesions in patients. As newer information becomes available, we will hopefully be able to develop highly specific methods to detect metastasis in patients which will greatly benefit overall patient outcome.

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Fig. 1.
Schematic diagram of the metastatic process.

Fig. 2.

Cancer-specific promoter-based imaging detects metastasis of human cancer in animal models. (A–D) Bioluminescent imaging (*BLI*). (E–G) Single-photon emission computed tomography (SPECT/CT). Melanoma (A and E), breast cancer (B and F), and PCa (PC3/ML) (C, D, and G) cells injected IV and developed as metastatic lesions in immunocompromised mice were imaged with reporter genes systemically delivered (IV) in an L-PEI nanoparticle under the control of tumor-specific promoters PEG-prom and AEG-prom. *v*, ventral; *d*, dorsal; *l*, left; and *r*, right views.

Fig. 3. Transcriptional amplification (TA) machinery. (A) Schematic diagram for the TA system. (B) In vitro enhancement of luciferase activity via the TA system. (C) In vivo sensitivity enhancement of TA vector. Note that TA system was able to detect small metastatic lesions (PC3/ML PCa) in liver and kidney (*lower images*), which were undetectable by the parental vector (*upper images*).

Fig. 4.

In vivo CEST-MR imaging of glioma expressing LRP. Rat glioma cell line (9L) and 9L-expressing LRP were injected left and right side of a brain of mice, respectively. Both CMV-prom (A and C) and PEG-prom (B and D) successfully drive the expression of LRP, which gives CEST contrast (C and D).