EBioMedicine 24 (2017) 34–42

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Review

MET Activation and Physical Dynamics of the Metastatic Process: The Paradigm of Cancers of Unknown Primary Origin

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ARTICLE INFO

Article history:
Received 24 July 2017
Received in revised form 5 September 2017
Accepted 18 September 2017
Available online 21 September 2017

Keywords:
Cancer
Metastasis
MET oncogene
Invasive growth
Mechanical forces
Rheology

ABSTRACT

The molecular and cellular mechanisms which drive metastatic spread are the topic of constant debate and scientific research due to the potential implications for cancer patients' prognosis. In addition to genetics and environmental factors, mechanics of single cells and physical interaction with the surrounding environment play relevant role in defining invasive phenotype. Reconstructing the physical properties of metastatic clones may help to clarify still open issues in disease progression as well as to lead to new diagnostic and therapeutic approaches. In this perspective cancer of unknown primary origin (CUP) identify the ideal model to study physical interactions and forces involved in the metastatic process. We have previously demonstrated that MET oncogene is mutated with unexpected high frequency in CUPs. We here analyze and discuss how the MET activation by somatic mutation may affect physical properties in giving rise to such a highly malignant syndrome, as that defined by CUP.

Contents

1. Introduction

Metastases identify a complex and multistep process which ultimately result in patients' death. During neoplastic progression, cells proliferate without control, loose cell-cell contact-inhibition, detach from their primitive sites and invade organs giving rise to secondary colonies (Hanahan and Weinberg, 2000). Cancer biologists have long understood that tumor progression towards malignancy derives from both genetic and epigenetic changes affecting a cell and the altered response from extrinsic soluble cues, such as growth factors, cytokines and chemotactic stimuli as well as secretion of soluble signals that facilitate matrix remodeling, angiogenesis and immune tolerance. Thus, tumor growth and metastases are intrinsically tied to the constituent cell's ability to sense, process and adapt to the mechanical forces in

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https://doi.org/10.1016/j.ebiom.2017.09.025

their environment. If each step towards malignant transformation is known to be characterized by a variety of genetic and histopathological checkpoints, it should be underlined that over the past two decades it has become evident that mechanical phenotype of both the cell and its surrounding stroma is equally important for tumor spreading and that it represents an element as crucial as genomic disruption and instability in the evolution of the tumor. Several data demonstrate that the driving force for metastatic spreading seems not to be based on the activation of metastasis-specific genes as late event in tumor progression (Jones et al., 2008) and that cancer cell dissemination can occur early during tumor formation (Hosseini et al., 2016). It is conceivable that aberrant activation of certain oncogenes could initiate dissemination before triggering primary tumor growth. Then, secondary lesions might become clinically evident after a dormancy phase (Harper et al., 2016) or even dominate the primary site growth (Wan et al., 2013). From this perspective, to understand the interaction between invading cancer cells and their 3D microenvironmental confinement and to clarify the role played by cytoskeletal stiffness and cell contractility regarding the invasive cell motility is mandatory to fully reveal the multifaceted scenario of cancer metastasis.

2. Cancers of Unknown Primary (CUPs)

In some instances, distant dissemination arises at an extreme early stage, so that metastatic phenotype reaches clinical relevance before the appearance of the primary lesion. These tragic cases define a highly malignant syndrome known as 'cancers of unknown primary' (CUP), representing 3–5% of the cancer population (Stella et al., 2012). Accumulated aggregate data from the last two decades have more than adequately demonstrated that CUP presentation could be extremely heterogeneous, with the hallmark being the clinical inability to find the anatomical primary site. Indeed CUP key features are: i) early dissemination meaning early apparent metastatic disease with no identifiable site of origin at the time of presentation; ii) clinical absence of primary at presentation; iii) unpredictable metastatic pattern; iv) aggressiveness. Based on morphologic presentation CUPs are properly defined as carcinomas, the vast majority of which (90%) is represented by adenocarcinomas less prevalent characterizations include squamous cell carcinoma and indifferentiated carcinomas. The inclusion of sarcomas, lymphomas and melanomas of unknown primary is sometimes reported, although in those cases the origin lineage is clearly solved (Pavlidis and Fizazi, 2009; Hainsworth and Fizazi, 2009). There are, at least, two different hypotheses which are trying to define CUPs: one regards them as a single unique molecular and biochemical basis (still to be defined) and responsible for 'disappearance' or 'dormancy' of the primary tumor and the upfront metastatic phenotype; the other suggests that CUP consist of unrelated groups of site-specific tumors which all share the property of a small primary which escape diagnosis (Varadhachary et al., 2008). Many efforts are now directed to integrate molecular-based medicine and clinical practice with the aim to assign a tissue of origin (ToO) (Greco et al., 2013; Hainsworth and Greco, 2014; Moran et al., 2016). On these bases, a further sub-classification has allowed the identification of predominant subtypes as gastrointestinal and gynecological carcinomas. However, histologic definition remains elusive (Oien, 2009; Varadhachary et al., 2008; Monzon and Koen, 2010) and at the present there are no clear theories regarding the CUPs biology, which is still almost unknown regarding the pathogenetic basis. Growing evidence highlight CUP as extremely heterogeneous tumor patters as every patient features a unique molecular asset. Very recent analysis from next generation sequencing allowed to identify two emerging combinatorial strategies targeting cell cycle bock either by epigenetic modifiers and by the MAPK/PIK3CA inhibition (Subbiah et al., 2017). On these bases, it is conceivable that CUP patients, rather to behave similarly to those patients who develop metastases from known primary tumors, require a truly personalized therapeutic approach tailored on their unique tumor fingerprint.

3. The MET-driven Invasive Growth Program in Metastasis and CUPs

Growing evidence sustains that metastasis follows the inappropriate activation of a genetic program termed '*invasive growth*', a physiological process that occurs during embryonic development and post-natal organ regeneration (Trusolino and Comoglio, 2002) The *MET* proto-oncogene is a key regulator of invasive growth. *MET* encodes for the tyrosine-kinase receptor for 'scatter factor' or Hepatocyte Growth Factor (HGF), a sensor of adverse microenvironmental conditions, e.g. hypoxia (Pennacchietti et al., 2003) and ionizing radiations (De Bacco et al., 2011) and drives cell invasion and metastasis through the transcriptional activation of the 'invasive growth signature', a genetic program including cell scattering, invasion, protection from apoptosis and angiogenesis (Fig. 1). We here plan to introduce the physics of the MET-driven invasive process, mainly focusing on how the mechanical dynamics of the program impact on biomolecular components in leading such an aggressive phenotype. This aim originates from our recent observation demonstrating that invasive growth is aberrantly activated - due to high frequency of *MET* gene somatic mutations - in CUPs (Stella et al., 2011). Thus, CUPs identify the ideal model to analyze how physical laws modulate metastatic behavior in transformed cells. In human cancers *MET* activation confers a selective advantage for tumor progression (Engelman et al., 2007). It generally occurs as a late event, mainly consequent to receptor overexpression driven by transcriptional upregulation; in some instances, overexpression is due to gene amplification (Comoglio et al., 2008). Somatic point mutations are rarely found, accounting for no more than 3–4% of unselected primary cancers (COSMIC database, www.cosmic.org). By a screening of about 50 CUP patients we have demonstrated, that *MET* is frequently mutated and active in CUPs. Because the hallmark of CUPs is their precocious neoplastic spread, it is likely that one or more molecular pathways involved in the metastatic process are hyperactive in these tumors. This notion implies that targeting invasive signals represents a strong rationale for therapeutic intervention. Moreover, based on CUP phenotype, it is arguable that cancer stem cells are at the root of CUP tumourigenesis and play a key role in CUP initiation, maintenance, and precocious spread. Recent evidence indicates that common molecular mechanisms control both invasive growth and stemness (Comoglio and Boccaccio, 2001) and suggests that MET may be involved in the concomitant regulation of both properties. In such a setting, we showed that the *MET* oncogene is frequently mutated (about 30% of cases, vs. the 3–4% of the general cancer population), in the absence of high mutational background. Nucleotide changes found clustered either in the kinase domain (TK) or in the extracellular semaphorin (SEMA) domain of the receptor. Mutated receptors were functional and sustained the transformed phenotype, suggesting that *MET* activating mutations are genetic markers associated with the CUP syndrome. All the mutations found occurred in fully undifferentiated carcinomas with no identifiable tissue of origin after exhaustive diagnostic evaluation. We defined this tumor population as *'truly CUPs'* featuring the most aggressive clinical phenotype. Within respect to the wild type receptor, TK and SEMA mutated cells feature an increased proliferation rate, motile phenotype, invasion capacity and anchorage independent growth potential both in basal condition and upon HGF stimulation. Whereas the tumorigenic potential of mutations activating the TK domain is well documented (Michieli et al., 1999; Graavel et al., 2004), the oncogenic potential of SEMA changes is somehow unexpected since they do not affect receptor phosphorylation. Thus, our data strongly suggest that the non-catalytic SEMA domain is involved in neoplastic invasiveness, although no clear mechanistic explanation is given by only biological characterization of SEMA mutants. Notably, we further demonstrated that the occurrence of SEMA mutations is associated with aggressive and radioresistant brain metastasis from known primary (lung) (Stella et al., 2016a, 2016b) thus confirming the tumorigenic potential of SEMA-mutated cells. Those findings allowed us to hypothesize that changes affecting the SEMA domain coding sequence may be reflected in structural alteration of the

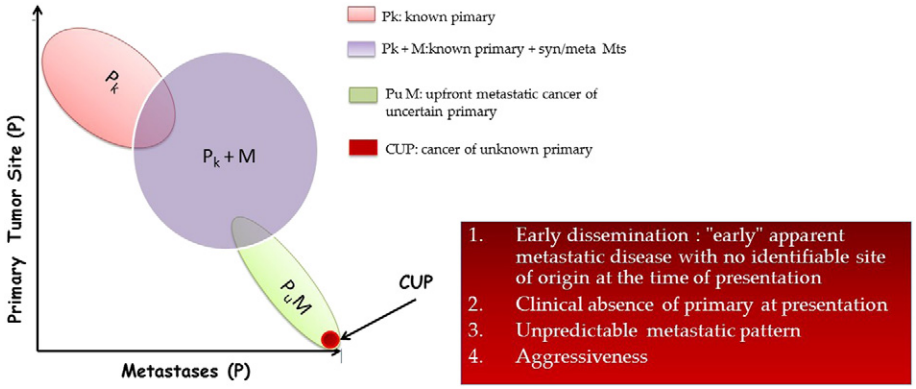

Fig. 1. Cancer of unknown primary (CUPs). Based on a graphic in which the axis of abscisses identifies metastatic growth and the axis of ordinates the primary mass growth, CUPs fall at the end (X max, Y = 0) on the continuum of cancer presentation.

extracellular portion of the MET receptor. This could, in turn, affect the physical interaction of CUP cells and surrounding microenvironment thus promoting their highly invasive properties. The unique SEMA-mutated CUP cell phenotype is essentially defined by the two following characteristics: i) the capacity to disseminate through the blood vessels which allows the randomic and multiple pattern of metastatic growth; ii) a specific viscoelasticity which provides the property to torsionally deform and cross anatomical obstacles such as the blood-brain barrier. Could the malignant advantage of SEMA mutated cells - as found in metastatic lesions of known and unknown origin - be related to their physical properties? We reasoned that the precocious metastatic spread of *MET* mutated CUPs can be sustained by nomadic cancer cells with stem-like features, in which pro-invasive signals are hyperactivated by physical forces and interactions. Indeed, although biochemical and biological features of these cells have been largely evaluated - also in our previous work - details of mechanical and biological interaction remain elusive. Nevertheless, the study of mechanical deformation of cells may provide key insights for understanding how the changes in cellular structure, response and function under forces may contribute to the invasive phenotype. More importantly, this approach may offer new opportunities for the diagnosis and treatment of such aggressive diseases.

4. Mechanical Phenotype of Metastatic Cells

Living cells - and cancer cells - can sense mechanical forces and convert them into biological responses. Most of the transformed cells feature variation in size and shape from 1 to 100 μm, (Emmelot, 1973) and comprise many constituents. The cell is covered by a phospholipid bilayer membrane reinforced with protein molecules, and the interior of the cell includes a liquid phase (cytosol), the nucleus, the cytoskeleton consisting of networks of microtubules, actin and intermediate filaments, organelles of different sizes and shapes, and other proteins. Notably, it has been demonstrated that primitive stem cells in adult tissues as well as cancer stem cells (CSC) are generally smaller than the normal differentiated elements (Li et al., 2015). This finding suggested a correlation between cell size and differentiation phenotypes and pointed out that cell size is correlated to CSC activity. Notably, CSCs can be compared to a gel microsphere embedded in a stiff and complex scaffold, being the sphere the 3D solid structure at lower energy. The study of how mechanical forces affect cellular function is termed *mechanobiology*, while how the forces affect ECM function is defined as *biomechanics*. In physiologic settings, cells pull on the extracellular matrix to move and need to be attached to it to duplicate. Nevertheless, proliferation decreases when cells come in contact according to the phenomenon called contact inhibition of growth. The interplay between the biophysical properties of a cell and the ECM establishes a dynamic reciprocity in which cell ability to exert contractile stresses against the extracellular environment balances the elastic resistance of ECM to deformation. This force balance is involved in regulation of a wide variety of cellular process, including invasive growth. Cells are exposed to a variety of mechanical forces and, on the other hand, exert mechanical tensions on their surrounding microenvironment. Rheology is the study of flow and deformation materials under applied forces. As many commonly-used material systems, living cells exhibit rheological properties, since - in contrast to ideal elastic materials -, their deformation depends on the rate of loading or better is strain-dependent, as for viscoelastic materials. Viscosity and viscoelasticity can vary upon external condition applied (e.g. stress, strain, temperature, timescale) and on internal variations such as protein concentration stability. Cellular models developed to study biomechanics derived either from a micron-nanostructured approach or by a continuous one (Lim et al., 2006). The first considers cytoskeleton as the most relevant structural component of the whole cell and it is mainly used to investigate biomechanics of adherent cells. On the other hand, according to the continuous approach the cell is a compressible material harboring homogeneous continuous properties and properly evaluates the mechanical properties at single cell level. Three continuous models have been developed for living cells: 1) cortical shell-liquid core (or liquid drop) models which consider the cell as homogeneous matter and do not take in consideration the cellular membrane; 2) solid models (elastic or viscoelastic) representing the cell as springs combined with pistons; 3) structural damping models which are in general obtained by using transient stress, such as creep or relaxation. Under physical perspectives, viscoelasticity is not plasticity, with which it is often confused. A viscoelastic material will return to its original shape after any deforming force has been removed (it will show an elastic response) even though it will take time to do so (it will have a viscous component to the response); on the contrary, a plastic material will not return to its original shape after the load is removed. Thus, when the viscous-elastic deformation presents after a stress threshold, we are in the presence of an elastic visco-plastic behavior. Nevertheless, few is known about single cells and- in particular - neoplastic scattering cells. It is reported that for an invasive tumor with individual invasive cells that detach themselves from the primary tumor and migrate into the surrounding microenvironment, a rougher tumor surface could imply that the individual invasive cells possessed a strong extracellular matrix (ECM) degradation ability, high motility and weak cell-cell adhesion (Jiac and Torquato, 2012). In conclusion, in addition to genetics and environmental factors, the physical properties and biomechanical between the arising metastatic sub-clone and its surrounding microenvironment are emerging as key determinants of each of the multiple phases characterizing the metastatic process. The deformability of cells is determined largely by the cytoskeleton, whose rigidity is influenced by the mechanical and chemical environments including cell–cell and cell–ECM interactions. During the complex metastatic process, cells detach from primary site, vascularized tumor, penetrate the surrounding stroma,

enter nearby the blood vessels and circulate in the vascular flow. Some of these cells eventually adhere to the blood vessel walls and are able to extravasate, migrate into local tissue where they can form secondary lesions (Fig. 2).

4.1. Detachment From the Primary Site

The detachment of a cancer cell from the epithelium and the subsequent invasion of surrounding stroma recall the well-characterized epithelial-to-mesenchymal transition (EMT), or - in other words - the invasive growth program. The latter is dictated by the physico-chemical properties of the ECM. In physiological conditions, cells exert and absorb continuous tensile traction forces on the ECM via their integrin attachments (Tan et al., 2003; Marchiò et al., 2012; Bartolomè et al., 2014). Even the stem cell population is sensitive to these mechanical cues Saha et al., 2008). Alteration of the mechanical interaction between cells and their surrounding stroma contribute to tumor onset and dissemination. As a tumor cell detaches from the primary mass and invades the surrounding parenchyma, its continues to exchange mechanical forces with its environment, among which tractional forces associate to locomotion and protrusive forces of the edge of the cell. The latter are associated to the formation of invadopodia which are active in facilitating initial digestion and invasion of the ECM (Yamaguchi et al., 2005a). Three-dimension (3D) microscopy studies of cancer cell motility documented that invadopodia are protruded first, pulling the rear of the cell forward. Cells inside a 3D matrix never push the surrounding matrix and only pull on surrounding fibres (Wirtz et al., 2011). This activity requires the activation of multiple actin binding proteins, such as cofilin, Arp2/3 complex and its activators neural Wiskott-Aldrich syndrome protein (N-WASP), Wiskott-Aldrich syndrome family, member 1 (WASF1; also known as WAVE1), WASF2 and WASF3 (also known as SCAR3) (Yamaguchi et al., 2005b). Their expression has been correlated to poor outcomes in several cancer types (Iwaya et al., 2007). Indeed, it has been demonstrated that transformed epithelial cells express different intermediate filament profiles and cytoskeletal architectures than their normal counterparts as demonstrated by the fact that vimentin filaments follow the pre-existing cytokeratin network during the epithelia-to-mesenchymal transition phase in metastatic cancer cells (Kokkinos et al., 2007; Pagan et al., 1996). Notably, cellular traction on collagen fibres may activate matrix metalloproteinases (MMPs). Therefore, the interplay between pulling by cell protrusions, MMP activity and remodeling of pre-existing matrix which support subsequent invasion, occurs within a feedback loop (Ellsmere et al., 1999). As discussed above, dynamic changes in cytoskeleton organization and cellular mechanics occur, thus determining variation in cellular and tissue stiffness. Growing evidence suggests that cancer cells feature lower stiffness than normal ones (Wirtz, 2009). In addition, tumor expansion induces compression of the surrounding ECM, which in turn constricts flow in vasculature, lymphatic system and interstitial space. When these compressive stresses occur in the setting of tissue that is highly compliant at baseline, such as pancreas or brain, altered organization and thinning of the basement membrane occurs. The latter, may be involved in the neoplastic disorganization as well as in the breakdown of boundaries which characterizes malignant invasion (Ingber et al., 1981). These compressive forces also contribute to clinical presentation of tumors, such as symptoms related to increase intracranial pressure or biliary obstruction in case on pancreatic cancer (Watanapa and Williamson, 1992; Schankin et al., 2007).

4.2. Intravasation and Circulation

During the journey through the circulatory systems, cancer cells are subjected to hemodynamic forces, immunological stress and collisions with host cells, among which blood cells and endothelial elements of the vessel walls. All the processes inducing tumor cells squeezing between endothelial elements could affect cell survival and their ability to metastatize. Moreover, they are known to be able to activate gene programs associated with cytoskeletal remodeling and altered cell-cell adhesion which ultimately induce reinforcement of cell structure and attachment to vascular wall (Davies et al., 2005). Only circulating tumor cells (CTCs) which overcome the effects of shear forces and immunosurveillance, will eventually, be able to give rise to distant lesions. Indeed, a minor fraction of CTC survive, whereas the vast majority die (Steinert et al., 2014). Two physical and mechanical parameters can influence cells fate into vessels are the following: the pattern of blood flow and the diameter of vessels which can affect mechanisms of intercellular cellular adhesion leading to cell arrest in larger vessels. Shear forces arise between adjacent layers of fluid of viscosity moving at different velocities. The velocity of a fluid in a cylindrical tube is maximum at the center and zero at the periphery, near the walls. Thus, relative velocities of parallel adjacent layers of fluid in laminar flow define the

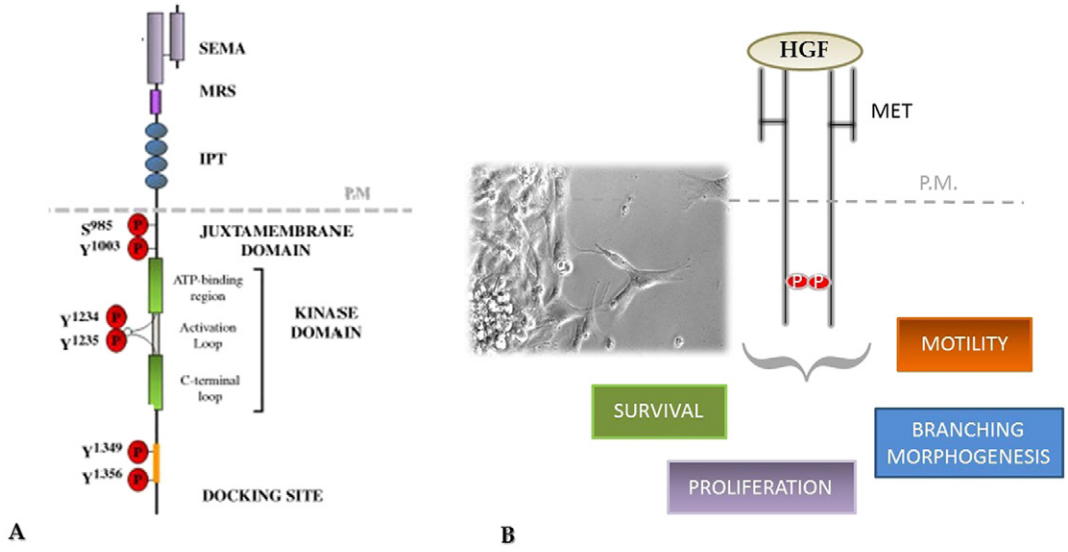

Fig. 2. MET and Invasive Growth. Panel A: schematic structure of the MET oncogene; Panel B: biological effects of the MET signaling activation cascade, namely the Invasive Growth program. SEMA domain, MRS: MET-related sequence domain, IPT domain: immunoglobulin-like structures; P: phosphotyrosine residue; Y1234 and Y1235: tyrosine residues at the catalytic site which regulate the enzymatic activity; Y1349 and Y1356: tyrosine residues at the C terminal regulatory tail that, when phosphorylated, create a unique docking site which is responsible for the recruitment of a wide spectrum of downstream transducers leading to Invasive Growth.

shear rate. The shear stress is defined by the product of viscosity and the shear rate. Shear stress influences translational and rotational motion of CTCs and determines thus modulating receptors-ligand interaction and cell adhesion. As to arrest, CTCs have to undergo two different mechanisms: i) physical occlusion: if the cell enters a vessels whole diameter that is less than its one, it can stop due to mechanical trapping; ii) adhesion: if the CTC is circulating a large diameter vessel, in order to arrest it needs to adhere to the vessel walls through formation of specific bonds. It has been calculated that the probability (P) of arrest at a large vessel can be calculated as $P \propto ft$, where f is the collision frequency between membrane-bound receptors and endothelial ligands and t is the residence time (Wirtz et al., 2011). The probability to arrest is expected to be maximum at intermediate values of shear stress. Moreover, it has been shown that shear can enhance phosphorylation of adhesion molecules in cancer cells thus enhancing adhesion to collagen-based ECM. Integrins and selectins cooperate in firming adhesions of cancer cells to ECM [(Hynes, 2002; Thomas et al., 2008), as well as adhesion molecules (Intracellular Adhesion Molecule 1-ICAM1 and Vascular Cell Adhesion Molecule 1-VCAM 1, E-cadherin) (Kostantopoulos and Thomas, 2009; Lee et al., 2017) and kinases (Focal Adhesion Kinases (FAK) in cancer cells (Xiong et al., 2017). Moreover, it has been reported that CTCs may escape tumor immunosurveillance and enhance their arrest, through association to platelets (Palumbo, 2005). The latter act on one hand by masking CTC from immune-mediated clearance and on the other (Nieswandt et al., 1999), by promoting cell adhesion to the vessel walls and by enhancing the release of vascular endothelial growth factor (VEGF) (Kim et al., 2007).

4.3. Arousal of Distant Metastasis

The pattern of metastasis has been explained by two hypotheses. According to “the seed and soil hypothesis”, originally defined by Stephen Paget, a tumor cell will metastasize where a local microenvironment is favorable (Paget, 1889), or better, the outcome of metastasis is determined by the complex interactions between cancer cells and their surrounding microenvironment. On the other hand, the mechanical hypothesis states that metastasis is likely to occur at sites based on the pattern of blood flow (Riihimaki et al., 2016). Both the hypotheses are thought to have complementary roles in influencing the arousal of metastatic lesions. Thus, when a tumor cell encounters a capillary which diameter is smaller than its one, ($d_{cell} > d_{vessel}$), the probability of cell trapping is very high. Then that cancer cell need to extravasate and to invade local tissue/organ and interact with local stroma before metastatic development. The probability of the occurrence of extravasation is directly related to the residence time after the collision between circulating cell and the blood vessel and by the expression level of receptor-ligands adhesion molecules. It has been reported that more than 50% of metastasis could be explained by the blood flow pattern between the primary and secondary site (Weiss, 1992). Notably, the level of shear stress corresponding found in venous system, has been calculated to be optimal to achieve sufficiently long residence time (Mc Carty et al., 2000). On the other hand, higher shear stress corresponding to that found in the arterial circulation, is associated to cell cycle arrest of circulating cancer cells which are removed by immune system elements Chang, 2008). As opposite, it has been reported that venous shear stress can induce EMT, as shown by the shear-mediated internalization of E-cadherin in metastatic cells from oesophageal cancer (Lawer et al., 2009). For a tumor cell to escape vasculature and colonize distant site, it must undergo diapedesis through the endothelial wall, which introduces additional mechanical interactions between the tumor cell and endothelial cells. Diapedesis is defined as process by which cells extend pseudopodial processes that penetrate cell-cell junctions in endothelium which requires local and dynamic chances in cellular mechanisms leading to a transition from cell-cell adhesion to cell-ECM adhesion. The mechanisms underlying this switch may include conformational activation of existing integrins and expression of newly recombined ones (Stewart et al., 2004). Degradation of ECM is necessary for tumor invasion and the most significant ECM component that can modulate cell motility through ECM are collagen, fibronectin (FN) and proteoglycan (PG). It has been reported that collagen can resists to tensile forces, while FN and PG resist to compressive forces. These latter components are easily digested by the most MMPs, whereas collagen is limited degraded by few MMPs, among which collagenases MMMP1, 8 and 13 as well as gelatinase (MMP2). It has been demonstrated that when collagen is stretched in tension it becomes resistant to enzyme cleavage (Wyatt et al., 2009). There is scientific evidence that the collagen in the stroma is stretched by the expanding tumor (Pasek et al., 2005; Provenzano et al., 2008). Thus, when the tumor expands, the collagen in the stroma will realign and stretch perpendicular to the expanding tumor to resist the tumor expansion and stroma degradation. On the other hand, the tumor cells need to overcome increased collagen alignment and stretching in order to invade the stroma. Indeed, it is known that cancer cells can increase their traction forces in response to increase ECM stiffness documented by their transition to a myofibroblastic phenotype (Solon et al., 2007; Hinz, 2010) and that they must also degrade ECM through synthesis of MMPs, specifically directed against collagen, PG and FN (Xu et al., 2005; del Casar et al., 2010).

5. Biomechanical Properties of MET Mutants: the Paradigm Shift.

The evolution of freely growing tumor is defined by an initial exponent growth followed by a transition period, during which growth becomes linear in time. If the tumor mass is assumed as spherical, in absence of external loads the change in growth rate is essentially due to the reduced availability of nutrients that occur when the diameter of the spheroid overcomes the diffusion length of the nutrients in the spheroid. Coherently, MET activation is mainly induced by hypoxia derived by discrepancy between tumor mass and tumor vasculature and nutrients availability. Therefore - in this scenario - MET-driven metastatic dissemination occurs as a late event (Trusolino and Comoglio, 2002). Quite opposite, the highly malignant CUP-cell phenotype is defined by the two following critical features: i) the capacity to early detach each other and to scatter; ii) the ability to easily cross anatomical obstacles such as endothelial wall or the blood-brain barrier. These points define - in essence - the *Invasive Growth*. How MET early activation by mutations contribute to the acquisition by the cell of the physical properties which are required for tumor spreading? As already mentioned, the model above described can be certainly improved by considering the viscoelastic behavior that characterizes the most biological materials. Thus, cells adhere to each other via cadherin junctions and to the ECM via integrin junctions. These bonds have a limited strength: the adhesive strength of a single bond has been found to be in the range of 35–55 pN. Since the density of E-cadherin on a cell surface is about 400–800 molecules/μm (Basilico et al., 2014) of the surface, one can estimate the resistance to pulling to be of the order of 0.1 kPa (Baumgartner et al., 2000; Canetta et al., 2005). If early scattering is the key feature of CUPs, it is reasonably conceivable that a proper cytoskeletal rheology allows the acquisition by the CUP cell structure of higher number of degrees of freedom. Therefore, the cell amplifies her response to central forces mainly derived by externally applied mechanical forces and starts to migrate into the elastic matrix. The effects of this interaction prevail on the expected capacity of the transformed cells to continue proliferating to form a multilayer by losing contact responsiveness. It has been already reported that MET controls actin cytoskeleton which in turn, promotes single cell scattering (Ponta et al., 2003; Menard et al., 2014). Some isoforms of the transmembrane cell adhesion molecule CD44 are known to link MET to the cytoskeleton thus modulating structural and topographical regulation at the inner side of the cellular plasma membrane. Within

respect to mutated *MET*, as found in CUPs, TK and SEMA mutation seems to act as functional genetic markers of the disease. Whereas in cells expressing *MET* TK mutants, actin is markedly modified through altered receptor endocytic trafficking (Joffre et al., 2011). Accordingly, the TK mutants loss actin stress fibres and remodel their cytoskeleton to induce migration. As opposite, no clear mechanism has been defined for SEMA mutants. Nevertheless, based on the above discussed data, it is clearly evident that SEMA mutations are associated to highly invasive phenotype, as well. SEMA-mutated cell feature increased migratory capacity if compared to *MET* wild type cells (Stella et al., 2011). Notably the reported rates were lower than those displayed by TK mutants. The SEMA-mutated cell motile behavior, measured by the transwell migration assay, seems to be not related to matrices stiffness and it not varies at different pore sizes (data not shown). This finding is quite unexpected, as we could instead hypothesize for CUP cells high migration ability even at smallest pore sizes, as described for highly aggressive cancers (Justus et al., 2014; Guzman et al., 2014; Anguiano et al., 2017). Although further experimental validation is required, our preliminary data point out that cell plasticity characterizing SEMA-mutated clones is not relying on increased deformability and adaptation to foreign environments, as expected (Chaffer and Weinberg, 2011). Transferring this concept to tumor mechanics, it is conceivable that a paradigm shift in the basic concept of tumor dissemination biology is required based on the necessity to introduce a pseudo-plastic behavior in the description of MET-related metastatic phenotype. According to this hypothesis, SEMA-mutated cells are subjected to a sufficient high tension or stress that is responsible to break bonds between cells and the ECM. In this way, duplicating cells do not find place among their neighbors, bounce off the ECM becoming prone to intravasation and distant dissemination (Fig. 3). To be more realistic, one should hypothesize that mutations affecting the extracellular SEMA domain, might result in an unexpected interaction between mutated cell and the ECM which allows cellular scattering. It should be taken into account that those cells mechanically behave as restless elements, unable to follow the performance of a freely growing tumor, as it happens in the vast majority of cases. The selective advantage of *MET* mutation is reflected in an altered ECM/cell stiffness balance might be responsible to the impaired cell ability to degrade ECM. The latter allows cells to spread towards blood vessels, where they are likely to follow the expected rheology behavior.

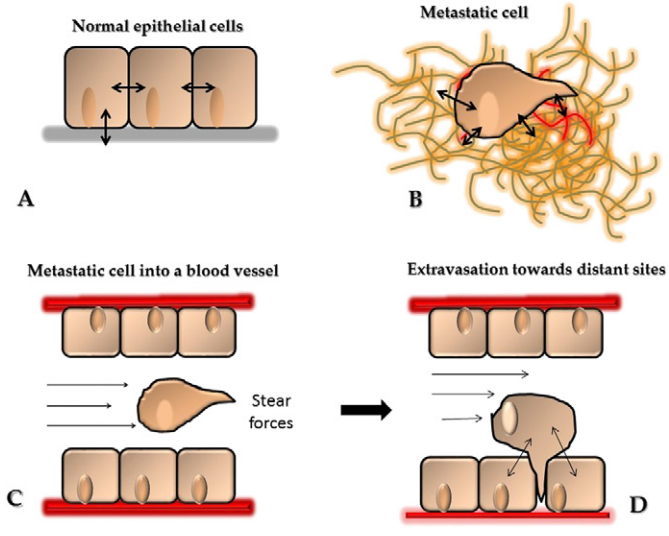

Fig. 3. Biomechanics of metastatic dissemination. Panel A: even in normal tissues, cells are subjected to mechanical forces: i) Receptor-receptor interaction, ii) Interstitial pressure; Panel B: continuous exchange of mechanical forces between tumor cell and surrounding environment: i) traction forces; ii) protrusion anterior forces; Panel C: when the tumor cell reaches the vasculature it is subjected to shear stresses associated to blood flow; Panel D: to cross the endothelium, the metastatic cell is exposed to more mechanical forces and viscoelastic interactions which precede the ECM transition.

6. Therapeutic Perspectives

Although *MET* genetic alteration/activation occurs in a minority of many cancer types and the mutational landscape of CUP can be extremely heterogeneous, the role of *MET* mutations in neoplastic spreading is well defined and documented. In this scenario, CUPs are paradigmatic since they could represent the ideal model to study the biological basis of the metastatic process through a continuum from neoplastic dissemination from occult primary to early metastatization from defined primary sites. Notably, *MET* genetic alteration is being targeted and in some instances quite successfully by various MET inhibitors. Several reports are already available on these topic (for a review see Comoglio et al., 2008; Gherardi et al., 2012; Stella et al., 2016b; Ma, 2017). Details on anti-MET agents which already landed the clinical arena are summarized in Table 1. MET inhibitors are represented either by monoclonal antibodies or small molecules. The latter are mainly developed as tyrosine kinase inhibitors (ATP or non-ATP competitive). Based on the hypothesis discussed above, we here we aim at focusing on the rationale of therapeutic blockage of MET SEMA domain. The latter structurally identifies the site of MET-ligand HGF binding and is necessary for receptor dimerization and activation (Kong-Beltram et al., 2004). Very few data are available on specifically effectiveness anti SEMA agents. Strategies to prevent MET dimerization with soluble SEMA domain showed to be effective in reducing receptor signaling thus suggesting that SEMA domain might not only be a promising anticancer target, but it can also behave as biotherapeutic per se (Kong-Beltram et al., 2004). The MET selective kinase inhibitor SU11274 has been reported to be capable of in vitro inhibiting HGF-induced signaling cascade activation through *MET* wild type as well as mutant receptors harboring mutation in the SEMA, juxtamembrane and tyrosine kinase domain. However, mutation in the SEMA domain resulted in varying response to the inhibitor (Jiang et al., 2007). Overall, the vast majority of agents developed as monoclonal antibodies can effectively target *MET* gene amplification, rather mutated receptors. Basilico and coworkers have previously identified 1 hotspot that coincided with the known HGFβ chain binding site on blades 2–3 of the SEMA domain β propeller. This spot could be effectively druggable by specific antibodies according to promising in vitro analysis (Basilico et al., 2014). The recombinant monoclonal antibody onartuzumab (MetMab) which could bind the SEMA domain, failed against lung cancer and has been stopped due to a lack of clinically meaningful efficacy in a Phase III study evaluating onartuzumab in combination with erlotinib in non-small cell lung cancers overexpressing MET by immunohistochemical staining (Perol, 2014). Although several criticisms can be responsible of study failure, the most relevant one was that receptor overexpression might not be the proper target for onartuzumab. The novel bioengineered bispecific EGFR-MET antibody JNJ-61186372 has an epitope that blocks HGF ligand binding at SEMA domain, but it is distinct from the onartuzumab epitope (Merchant et al., 2013). In preclinical animal models, the antibody can successfully modulate both EGFR and MET axis in EGFR inhibitor-resistant NSCLC (Moores et al., 2016). The very recently developed IgG2-enhanced next generation MET monoclonal antibody KTN0073 exhibit potent antitumor properties both in vitro and in vivo on both MET amplified cells but also in the juxtamembrane exon 14 deletion mutants (Yang et al., 2016). The novel phage-derived anti-MET antibody, 7A2/107_A07 which competes for MET ligand HGF as well as for the HGF fragment NK1 by binding the IG1domain of the receptor rather than the SEMA domain (DiCara et al., 2017) (Fig. 4).

In conclusion, it is clearly evident that very sporadic attempts have been focused on targeting MET SEMA domain. Notably all the SEMA inhibitors already clinically available can target *MET* amplification rather than somatic mutations. Some studies indicate that SEMA mutations may be associated with higher HGF affinity and that MET E168D mutants may show increased sensitivity to MET inhibitors. However, the activity of anti-MET drugs in non-kinase domain-mutated tumors

Table 1
Details on clinically available MET inhibitors.

Agent	Target/action	Cancer type
Anti-MET monoclonal antibodies		
SAIT301	Ig-like extracellular domain	Advanced MET positive solid tumors
ARGX-111	HGF competitor	*MET* amplified cancers
Onartuzumab	SEMA domain	Advanced/metastatic solid tumors
JNJ-61186372	SEMA domain (different epitope from onartuzumab); MET-EGFR bispecific Ab	Advanced NSCLC
ABT-700	HGF competitor/MET density	Advanced *MET* amplified solid tumors
MET tyrosine kinase inhibitors		
Crizotinib (PF-02341066)	Triple kinase inhibitor (MET, ALK ROS1) -*MET* amplification	Advanced NSCLC, gastric cancer, metastatic urothelial cancers, anaplastic large cell lymphoma, CRC, advanced/relapsed/refractory solid tumors, primary CNS tumors
Cabozantinib (XL-184)	Triple kinase inhibitor (MET, RET, VEGFR2)	NSCLC with brain metastases, advanced cholangiocarcinoma, metastatic triple negative breast cancer, CRC, metastatic Merkel cell carcinoma, recurrent endometrial cancer, breast cancer with brain metastases, metastatic renal cell carcinoma
Volitinib/Savolitinib (HMP504/AZD6094)	*MET* amplification	Gastric adenocarcinoma, papillary renal cell carcinoma
Foretinib (GSK13630089)	TK domain (ATP-competitor)	Papillary renal carcinoma, medulloblastoma, metastatic gastric cancer, hepatocellular carcinoma
AMG337	*MET* amplification, *MET* mutation (Y1230 and D1228) in vitro	Advanced gastric and aesophageal adenocarcinoma, advanced solid tumors
Tivantinib (ARQ-197)	Non-ATP competitive TK inhibitor	Relapsed/refractory multiple myeloma, locally advanced or metastatic CRC, metastatic triple negative breast cancer, childhood relapsed/refractory solid tumors, advanced head and neck cancers, gastric cancers, metastatic solid tumors, mesothelioma, SCLC, HCC
Capmatinib (INC280)	*MET* amplification, *MET* Ex 14 skipping mutation	NSCLC, CRC, HNSCC, advanced solid tumors, HCC, metastatic CRC, metastatic renal carcinoma, recurrent glioblastoma, advanced metastatic melanoma
EMD1204831	ATP-competitive inhibitor	Advanced solid tumors, advanced HCC
Glesatinib (MGD265)	*MET* (amplification, ex 14 mutation), AXL dual inhibitor	Advanced solid cancers
MK8033	MET/RON TK domain, ATP competitor	Advanced solid tumors
PF-04217903	MET TK domain, ATP competitor	Advanced cancers

NSCLC stands for non-small cell lung cancer, SCLC: small-cell lung cancer, HCC: hepatocellular carcinoma; CRC: colorectal cancer, HNSCC: head and neck squamous cell carcinoma, CNS: central nervous system; EGFR: epidermal growth factor receptor, VEGFR: vascular endothelial growth factor receptor, ALK: anaplastic lymphoma kinase, ATP: adenosine triphosphate.

remains to be fully elucidated. The lack of mechanistic correlation between biomolecular and clinical findings is most probably due to the fact the tumorigenic potential of the SEMA domain is not fully deciphered. In this setting, the paradigmatic shift discussed above, sustains a rationale for more extensive efforts in developing anti-SEMA agents. Therefore, by therapeutically acting on MET SEMA domain activation, the biomechanical properties of metastatic *MET*-mutated clones might be re-modulated towards a restored ECM/cell stiffness balance. The latter could cooperate in generating a less aggressive malignant phenotype.

7. Concluding Remarks

Cancer cells, like living organisms are far more complex than engineered materials: they are dynamic and provide integrated functions which allow their proliferation and progression. It is becoming

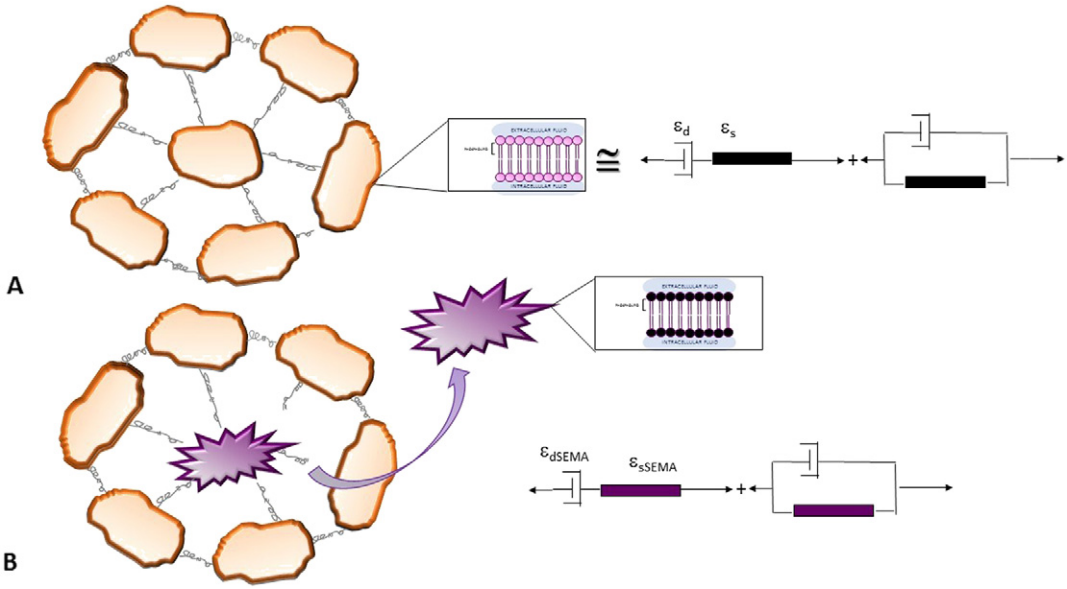

Fig. 4. Schematic representation of visco-elastic behavior in MET-driven tumor spreading. Panel A: cancer cells can be equated to visco-elastic materials, which behavior is described by a combined model deriving from the Maxwell and Kelvin-Voigt arrangements of the spring (εs) and dashpot (εd) at the plasma membrane level; Panel B: by the occurrence of MET SEMA mutation, the forces in both spring (εsSEMA) and dashpot (εdSEMA) change. The latter induces the break of cell-cell bonds thus promoting cancer cell scattering and dissemination which becomes prevalent on tumor mass growth.

clearer and clearer that, in addition to biochemical signaling, the mechanical interaction of the tumor with its surrounding environment plays an important role for tumor onset and metastatization. In other words, cancer cells can sense mechanical stress and tensions and convert them to biological signals, ultimately leading to malignant phenotype. Although mathematical and physical modeling has been applied to deeper understand primary tumor growth, little is known about mechanics of metastatic cells. Cancers of unknown primary site define the optimal prototype to characterize the mechanical behavior of metastasis. In this respect, the MET-driven invasive growth deserves great attention since it orchestrates the biological program leading cells to distant dissemination. *MET* mutations have been found in CUPs being clustered to the SEMA and TK domain of the receptor. The biomechanical properties of *MET* mutants might trigger the hyper-invasive phenotype associated to CUP. We are proposing that the structural resistance of a MET-mutated cell to deformation might be at the same time, its selective advantage leading to the arousal of secondary lesions, and the physical validation of a specific malignant phenotype. This new postulate defines a shift in the biomechanics previously applied to cancer cells, centered on the concept that forces of deformation push cell dissemination. This crucial point may have the potential to open novel research focuses towards a deeper understanding of tumor progression with relevant diagnostic and therapeutic implications.

Search Strategy and Selection Criteria

Data for this Review were identified by searches of MEDLINE, Current Contents, PubMed, and references from relevant articles using the search terms “invasive growth”, “MET oncogene”, and “CUP” “biophysics”. Only articles published in English between 1980 and 2017 were included.

Funding

AIRC IG, Project no 15572 - AIRC Special Program 5xMille 2010 MCO, Project no 9970.

Acknowledgements

The authors thank Andrea Marchelli for fruitful discussion and constructive comments and Ernesto Pozzi for critical reading of the manuscript.

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