Chapter 5

Early Metastasis in Colorectal Cancer Poses an Option for New Diagnostic and Treatment Strategies

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Additional information is available at the end of the chapter

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Abstract

Metastasis is the spread of tumor cells from a primary site to a secondary site within the host's body. It is initiated by the detachment of the tumor cells from the primary tumor followed by invasion into the surrounding tissue. Thereafter the cells migrate across the endothelium and into the blood vessels (intravasation). During the intravasation the cells have to survive the sheer forces and the immune response. Upon arrival to the target organ, the cells leave the circulation and cross the endothelium to reach the host organ. Once there, the tumor cells are greeted with the organ's local immune cells and with a hostile or inappropriate environment, where they finally have to form proliferating colonies. Metastasis is therefore far from being a straight-forward or efficient process with less than 0.1% of disseminating tumor cells (around 1 × 109 cells per day for a 1 cm size tumor) succeeding in colonizing distal organs. The identification of the involved marker during the early metastasis process will be essential for establishment of new diagnostics tools, as well as development of novel treatment strategies.

Keywords: colorectal cancer, early metastasis, migration, invasion, homing

1. Introduction

Cancer metastasis is the major cause of cancer morbidity and mortality, and accounts for about 90% of cancer deaths [1]. Metastasis is a complex process requiring several processes, which involves the spread of cancer cells from the primary tumor to surrounding tissues and to distant organs. Cancer cells require the capacity to invade the surrounding tissues, then

migrate and survive in the circulation, colonize the foreign organ and eventually resume growth, to gain metastatic capability [2], Metastasis alone is an inefficient process because the tumor cells have to acquire some necessary abilities to regenerate a tumor at a distant site [3, 4]. Over the past few years, the main determinants of metastatic competence in CRC have begun to be characterized. In fact, the acquirement of a stem-like phenotype by cancer cells is very important for the tumor cells to regenerate in a foreign organ, in the absence of mutations associated with the metastatic process in CRC. These metastatic stem cells adopt multiple phenotypes and behaviors and critically depend on their interaction with the microenvironment to migrate, survive in the circulation and flourish in a foreign organ. The metastatic cascade consists of four essential steps. The first of which is the detachment of the tumor cells from the primary tumor. There after the separated cells undergo local invasion into the surrounding tissue, e.g. into the mesenchyma, followed by migration of the tumor cells across the endothelium and into the vessels in a process known as intravasation where disseminating tumor cells have to survive the circulatory system's sheer forces and swarming immune cells. Upon arrival to the target or appropriate organ, tumor cells have to leave the circulation and cross once again the endothelium to reach the host organ. Once there, the disseminated tumor cells are greeted with the organ's local immune cells as well as with a hostile or inappropriate environment where they finally have to form proliferating colonies. Metastasis is therefore far from being a straight-forward or efficient process with less than 0.1% of disseminating tumor cells (around 1×10^9 cells per day for a 1 cm size tumor) succeeding in colonizing distal organs. The cells have to face multiple ordeals, such as the immune system, at each step of the process thus making metastasis a process possible mostly out of the sheer number of disseminating cells entering the bloodstream [5]. Multi-biochemical events and other parameters affect the metastatic cascade such as extracellular matrix structure, growth factors, chemokines, matrix metalloproteinases. Hence, the biochemical markers along with the tumor microenvironment may serve as a crucial target for the inhibition and prevention of metastasis [5].

2. Metastasis in colorectal cancer

2.1. Cancer cell detachment

Cancer cell detachment is a process usually occurring from the extracellular matrix (ECM). Cell detachment involves both mechanical forces and protease-mediated cleavage, but also decreased expression of adhesion molecules and changes in glycosylation of cell membrane glycoproteins and proteoglycans. Mechanical forces are generated by actomyosin-driven contraction. The cytosolic dissociation of cell–substrate adhesions can also be performed by the calpain cysteine proteases, by phosphorylation/dephosphorylation of cytosolic adapter proteins and by posttranslational modification of integrins or adapter proteins.

Extracellular dissociation of cell-substrate adhesions can be achieved by proteolytic cleavage of matrix constituents that are mediated by matrix proteases. Moreover, the detachment

could occur by the shedding of matrix receptors such as integrins [6]. Anoikis is a form of programmed cell death that occurs when anchorage-dependent cells detach from their ECM [7]. When cells are detached from the ECM, there is a loss of normal cell-matrix interactions, and subsequently anoikis can occur through the down-regulation of Bcl-xL (an anti-apoptotic component of the mitochondrial pathway) and the up-regulation of Fas ligand (FasL) (an activator of the death receptor pathway) [8].

However, during the metastatic detachment, the tumor cells resist and escape the anoikis process. This escape includes the alteration of enzyme systems in the signaling pathways that regulate anoikis, such as small GTPases and effectors, receptor tyrosine kinases and other kinases such as NF-κB, and EMT factors [6, 9].

Furthermore, there are multiple anoikis-independent mechanisms by which normal epithelial cells would die once detached from the ECM. Metastatic cancer cells must overcome these anoikis-dependent and anoikis-independent barriers in order to survive once they lose the attachment to the ECM [6].

2.2. Detachment in mCRC

Metastatic colorectal cancer (mCRC) is a long and complex process involving several mechanisms, and molecular pathways. CRC is currently considered the third most common neoplasm in the world according to the World Cancer Research Fund International, and the second most frequent malignancy causing death [10] mCRC is a multi-step biological process (**Figure 1**). This process starts with a series of mutations in colonic epithelial cells, continues with their detachment from the large intestine, dissemination through the blood and/or lymphatic circulation, attachment to the hepatic sinusoids and interactions with the sinusoidal cells, such as sinusoidal endothelial cells, Kupffer cells, stellate cells, and pit cells. The metastatic sequence terminates with colorectal cancer cell invasion, adaptation and colonization of the hepatic parenchyma. All these events are termed the colorectal cancer invasion-metastasis cascade, which includes multiple molecular pathways. The cellular and molecular pathways of the metastatic process in CRC have been extensively analyzed over the last decades. The metastatic process in CRC involves a series of steps such as:

1. Lysis of the extracellular matrix

Enzymes produced by cancer cells alter the extracellular matrix and thus enable cancer cells to leave the original site of the primary tumor.

Matrix metalloproteinases (MMPs) are crucial components of cells that can degrade a range of extracellular matrix proteins allowing cancer cells to detach and migrate. MMPs are a family of zinc-dependent endoproteinases with an enzymatic activity directed against main Extra Cellular Matrix (ECM) components.

The mechanism by which MMPs aid cancer cells to escape and degrade the ECM consists of two main steps. First, proteinase acts by removing any physical barriers to invasion by the degradation of ECM macromolecules such as collagens, laminins, and proteoglycans. Second,

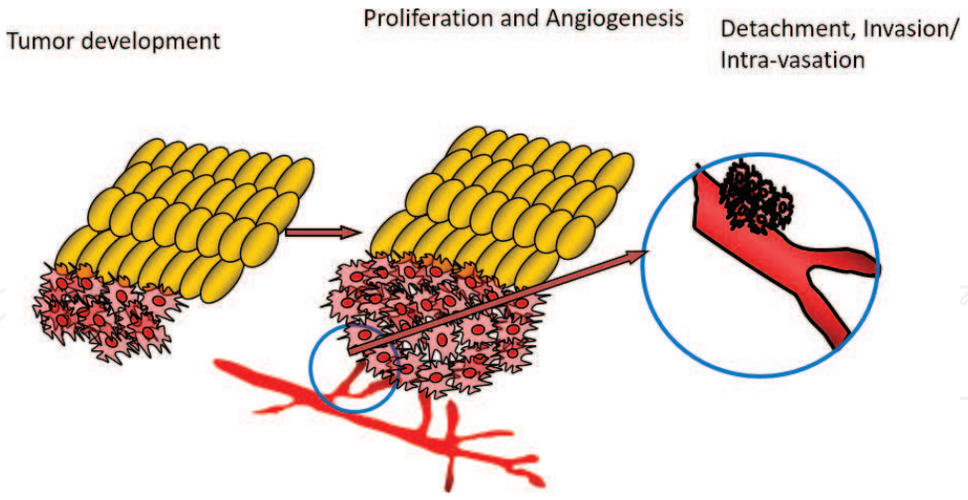

Figure 1. Illustration of the initial stages of the metastasis process. The tumor development is followed by tumor growth and angiogenesis. This stage is characterized by the down-regulation of cell attachment proteins such as the cadherins, as well as the tight junction proteins such as the claudins. In addition the expression of the matrix metalloproteinase is increased in this stage.

MMPs modulate cell adhesion. For cells to move through the ECM, they must be able to form new cell–matrix and cell–cell attachments and break existing ones. Overexpression of MMP-1, -2, -3, -7, -9, -13, and MT1-MMP has been demonstrated in human colorectal cancers (**Figure 1**). The degree of overexpression of some MMPs has been noted to be correlated with different stages of disease and/or prognosis [12].

2. Cellular adhesion

Cancer cells express adhesion molecules as cadherins, integrins, and carcinoembryonic antigen (CEA) that favor their adhesion to the extracellular matrix. These adhesion molecules have been under exclusive research regarding their roles concerning their regulation and expression in the process of early metastasis during detachment from the primary tumor.

A. Cadherins

Cell adhesion molecules (CAMs) regulate cell–cell and cell-matrix adhesion and are implicated in almost all stages of metastasis, therefore alterations in normal levels of CAMs such as E-cadherin will be significant in tumor progression. E-cadherin is the prototypical member of the type-1 classical cadherins and is found at adherens junctions (AJs), which are structures that mediate cell-cell interactions. E-cadherin is a single-pass transmembrane glycoprotein containing five extracellular repeats that mediate its Ca^{2+}-dependent homophilic interaction with opposing molecules on neighboring cells [11].

Studies exploring the expression of E-cadherin and α-catenin in tumor tissues have shown that loss of both molecules is linked to an increased invasiveness of tumor cells [12]. Evidence for this comes from in vitro and in vivo studies, which demonstrate that E-cadherin expression is inversely correlated with the motile and invasive behavior of tumor cells and also with metastasis in cancer patients [12]. Further studies have revealed that the relocalization of

β-catenin to the nucleus correlates with the acquisition of the mesenchymal phenotype [13], and is associated with the loss of E-cadherin.

Loss of the E-cadherin molecule is thought to enable metastasis by disrupting intercellular contacts. This can occur due to somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the *CDH1* promoter. Silencing can also occur either by DNA hypermethylation or through the action of transcription factors such as Slug, Snail, and Twist [14].

B. Integrins

Integrins are heterodimeric cell-surface glycoproteins that serve to mediate cell–ECM interactions, thereby linking cues from the extracellular environment to the actin cytoskeleton [15]. These membrane-spanning proteins consist of 2 subunits, termed a and b, of which there are at least 18 a-subunits and 8 b-subunits. The resulting multitude of possible combinations gives rise to more than 20 different integrins, which act to differentially control a range of biological processes through selective binding to extracellular substrates.

The crosstalk between epithelial cell-cell adhesion and cell- matrix adhesion signaling, and the dynamic interplay between the two, contribute to the plasticity within tumor cells that allows them to respond to external cues, which in turn drives effective migration and invasion. Below, we will review data on the key signaling intermediates that regulate this crosstalk, as well as discuss recent work that is in support of a physical interaction between integrin- and E-cadherin-mediated adhesions that governs the adhesive strength of E-cadherin.

C. EMT

Epithelial-mesenchymal transition (EMT) is a reversible morphogenetic biological process that involves the transition from stationary polarized epithelial cells to motile, multipolar or spindle-shaped mesenchymal cells. The EMT is described in detail in another part of this chapter.

2.3. Hypoxia

Hypoxia in cancer cell metastasis is a tumor oxygen deficiency termed as the environmental stressor (**Figure 2**). This condition is known to induce genes involved in the regulation of cell proliferation, extracellular matrix production, cell adhesion, and other hallmarks of tumorigenesis [16].

The mechanism behind the hypoxia induced metastasis is influenced by transcription factors like the hypoxia-inducible factor (HIF) family. This family consists of three members, HIF-1, -2, and -3, that regulate vital cellular processes such as glucose metabolism, angiogenesis, cell proliferation, and tissue remodeling [17].

Hypoxia is linked to tumor early metastasis by several known molecular mechanisms. The first mechanism is the HIF-1α binding to hypoxia-response elements within the c-met promoter activating transcription of this gene. Overexpression of the Met protein on the cell surface of tumor cells leads them to be more susceptible to hepatocyte growth factor stimulation. This causes extracellular matrix degradation, cell dissociation, and escape from hypoxic areas

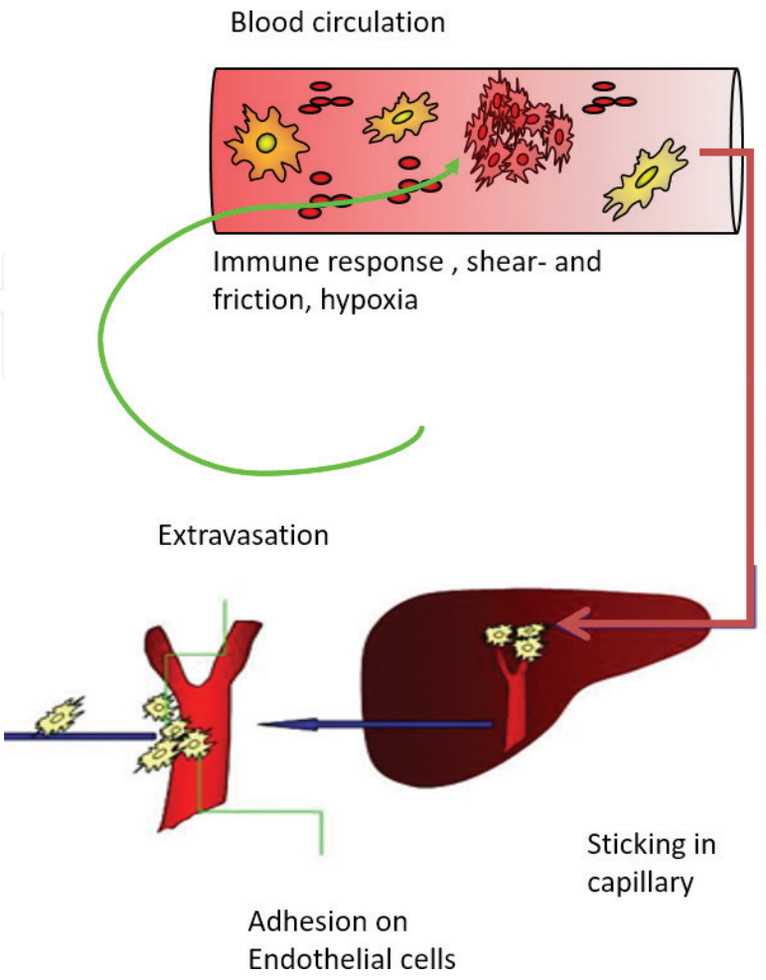

Figure 2. The post tumor cell intravasation stage poses the greatest obstacles for the tumor cells in the metastasis process. The immune response, shear and friction forces as well as hypoxia are responsible for considerable highest elimination of the tumor cells. The EMT is a special feature that defined the tumor cells in this time period.

to more oxygen-rich environments at a secondary [18]. Moreover, the HIF expression is necessary and sufficient to cause E-cadherin loss, a critical step in EMT [19].

While hypoxia has been strongly linked to tumor metastasis and poor clinical outcome of patients, it seems to actually have a dual role: insufficient oxygen limits tumor cell division while at the same time selecting for more malignant cells and inducing cell adaptations allowing for more invasive behavior. This is likely because low oxygen tension is able to increase cell invasiveness, cause cells to switch to anaerobic metabolism, increase genetic instability, and promote angiogenesis [20].

2.4. Invasion and endothelial transmigration

2.4.1. Mechanisms of invasion in CRC

The colonic epithelium in particular is composed of polarized cells with a characteristic apical membrane, forming a barrier with the components of the colon lumen, a basal membrane attached to the basement membrane and lateral membranes attaching to adjacent cells. In order

to invade other tissues, tumor cells may move individually, as clusters or as collective sheets by changing their phenotype and morphological features either by epithelial to mesenchymal transition (EMT), collective to amoeboid transition (CAT) or mesenchymal to amoeboid transition (MAT). EMT is the process of transition of the tumor cells from an epithelial phenotype into a mesenchymal phenotype by losing E-cadherin and upregulating vimentin [21]. This mechanism of invasion has been observed in colorectal cancer (CRC), breast cancer, hepatocellular carcinoma, pancreatic cancer, prostate carcinoma and lung cancer cells. CAT involves individual tumor cells detaching from cell clusters and developing amoeboid migration such as in melanomas. MAT describes the transition of mesenchymal tumor cells to amoeboid cells as observed in fibrosarcomas, melanomas and breast cancer. Amoeboid cells decrease their interactions with the extra-cellular matrix (ECM), which allows them to move easily through intact ECM gaps without resorting to proteolysis or ECM degradation and thus independent of protease activity [22]. EMT is the mode of invasion most observed and described in CRC.

2.4.2. EMT

EMT was first described as a process in developmental biology in embryogenesis. It was observed that a similar mechanism was employed by invading tumor cells, which undergo several phenotypical changes to resemble mesenchymal cells. This process requires the loss of cell–cell interactions such as the loss of epithelial cadherin (E-cadherin), α-catenin, claudins [23], occludin and ZO-1, with transcription factors' activation increasing the expression of mesenchymal proteins such as neuronal cadherin (N-cadherin), fibronectin and vimentin, reorganization of the cytoskeleton and production of proteases and ECM degrading enzymes [24]. The entirety of these processes is linked together, starting with the loss of E-cadherin. It binds extracellularly with its adjacent cell's E-cadherin while it binds intracellularly to α- and β-catenin and p-120 catenin, which is responsible for signal transduction and connecting the junctions to the cytoskeleton. Factors that have been known to induce EMT include transforming growth factor β (TGF-β), which plays an essential role in the transition and progression by activating Smad, integrins, platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). They induce the expression of EMT-related transcription repressors such as Snail, Slug, Twist and zinc finger E-box binding homeobox 1 (ZEB1). The downregulation of E-cadherin is generally accompanied by the upregulation of mesenchymal proteins, notably N-cadherin. This transition from E- to N-cadherin is called "cadherin shift" and is essential in EMT [25]. Upon loss of E-cadherin, the membrane-bound β-catenin is translocated to the nucleus where it regulates several genes' transcription, notably cyclin D1 and c-myc thus contributing to the progression of malignancy. In turn, N-cadherin expression mediated the formation of Rho-induced stress fibers forming lamellipodia (major actin projection leading the cell forward) by Ras-related C3 botulinum toxin substrate 1 (Rac1) protein activation and filopodia (thin actin projections from the leading edge of the cell) by cell division control protein 42 homolog (Cdc42) activation. Therefore, invading tumor cells undergoing EMT are able to detach themselves from clusters of epithelial cells; they lose their epithelial phenotype and are capable of moving individually similar to a mesenchymal cell.

Furthermore, EMT is known to preserve stem cell properties, evade apoptosis and the immune response as well as confer resistance to radiotherapy and chemotherapy, which was described in CRC. As of late, various microRNAs (miRs) have been shown to regulate EMT such as miR-9, which interacts with E-cadherin thus facilitating cell detachment and motility and increases VEGF levels leading to neoangiogenesis. Interestingly, epithelial cell differentiation may be driven by the miR-200 family including miR-200a, miR-200b, miR-200c, miR-429 and miR-141. Zinc Finger E-Box Binding Homeobox 1 and 2 (ZEB1 and ZEB2) transcription factors repress the miR-200 family at transcription, while this miR family itself inhibits ZEB1 and ZEB2 (EMT inducers) at a post-transcriptional level. Furthermore, ZEB induces EMT and stem cell characteristics by upregulating Sox2, Klf4 and Bmi1, which are ordinarily inhibited by the miR-200 family. This process has been conjectured to occur in CRC [26].

2.4.3. Collective cell invasion

Epithelial cancers such as breast and colorectal cancers witness the occurrence of collective cell or bulk invasion. The cells maintain their intercellular junctions, adherence and desmosomes, and thus stay attached together during movement [27]. However, there is a front to back polarity where tip or leader cells at the front of migration are phenotypically different than following cells. The asymmetry in actomyosin filaments affecting the tip cells are mediated by Rho GTPases and myosin II. Tip cells therefore become more similar to a mesenchymal cell as opposed to the following cells which maintain an epithelial phenotype with intact intercellular contacts. Cell movement occurs due to coordination in the polar CRC cells' cytoskeleton generating traction [28]. In order for movement to be possible, ECM and BM remodeling is required [29]. Stromal-cell derived factor (SDF1/CXCL12), FGF and TGF-β family are factors known to provoke collective cell migration. A two-dimensional invasion by a monolayer of cells, three-dimensional invasion or detachment of a cell cluster from the primary tumor are, among others, all different possible types of collective cell invasion (**Figure 2**).

In order to pull cells at the front and push those at the back, it is crucial to generate traction force. This is done by integrins present in the tip cells such as β1 and β3 integrins expressed therein, which attach to constituents of the ECM such as fibronectin by focal adhesion complexes [27]. Leading cells also express $\alpha 2\beta 1$ integrins, which bind to collagen and $\alpha v\beta 3$ integrins, which attach to fibrin. Following cells also form lamellipodia underneath the cells at the front whose $\alpha 6\beta 1$ integrins attach to the BM formed by the invading leading cells paving the way. Integrin binding to the ECM causes cytoskeletal changes such as activation of contractin, talin, paxilin and vinculin, which are cytoskeletal adaptor proteins. This induces actin reorganization, which is important for the formation of filopodia. Pseudopodia are regulated by Rac while filopodia are regulated by Cdc42. On the other hand, Rho is mainly involved in individual rather than collective cell invasion [30].

ECM degradation and remodeling is crucial for paving the way for migrating cells and is highly dependent on ECM density, gap size, orientation and dimensions as well as on the migrating cell [22]. Leading cells produce membrane type 1 matrix metalloproteinase; MMP14 (MT1-MMP), which degrades the initial outlet for movement through the ECM, which is then widened by the following cells. The latter continue ECM degradation followed by the production and deposition of laminin, perlecan, nidogen 1 and collagen type IV [31].

2.4.4. Mesenchymal cell invasion after EMT

In carcinomas, mesenchymal cells mainly originate from clusters of epithelial cells, which have undergone EMT. They generally invade as single cells [27]. Mesenchymal cell migration is a five-step process starting with pseudopodia formation at the front of the cell, which initiates focal contact with the ECM. Then focal proteolysis is followed by the contraction of the actomyosin filaments to pull the cell forward, which is finally followed by detachment of the trailing end from the ECM in order for the cell to be pulled forward [32]. The mesenchymal cell is partially polarized due to reorganization of the cytoskeletal F-actin resulting in a front able to bind tightly to the ECM and a trailing end or a tail that contracts refraction fibers in order to move [33].

TGF-β and nuclear accumulation of Smad2 were found to be the primary culprit in the detachment and de-differentiation of single tumor cells from moving epithelial clusters. On the other hand, it was found that Smad2 was retained in the cytoplasm in collectively invading cells and non-moving cells. Interfering with TGF-β type II receptor hindered intravasation or hematogenous metastasis for individual mesenchymal cells but tumor cell clusters moving by collective invasion were still observed in the lymphatic system [30]. Therefore, this study showed that TGF-β is crucial for individual cell movement via activation of the EGF receptor, fibrinogen/angiopoietin-related protein (FARP), E3 ubiquitin-protein ligase (Nedd4), Myosin phosphatase Rho-interacting protein (M-RIP), Smad4 and RhoC. However, it is necessary that TGF-β is downregulated thereafter to allow for tumor cell adhesion and subsequent colony formation at distal sites [12].

2.4.5. Role of TME

EMT is promoted by TGF-β, HGF, FGF, endothelial growth factor (EGF) and insulin-like growth factor (IGF). Along with interleukin (IL)-1α, these factors act on the tip cells and activate collective cell invasion. TGF-β in particular plays a major role in invasion by inducing EMT, and myofibroblast formation, as well as by producing autocrine mitogens and targeting CD8+ T cells to evade the immune response [34].

MMPs are upregulated in most cancers and are related to enhanced tumor growth, angiogenesis, invasiveness and metastasis. They are secreted from tumor cells, leading cells from collective invading cells, myofibroblasts and immune cells. They are central in the degradation of ECM proteins, the cleavage of cellular adhesion molecules like E-cadherin and the activation of cytokines and growth factors [35].

Immune cells from the TME were found to play tumor-promoting functions as demonstrated the observation that chronic inflammation often leads to the development of cancer, as for chronic Hepatitis C infection which causes hepatocellular carcinoma by its sustained and persistent inflammation. NF-κB is secreted by tumor cells and tumor-associated immune cells. It induces expression of the inflammatory cytokines IL-1, IL-6, TNF and RANKL, which stimulate invasion and metastasis. Tumor cells can actively regulate the tumor microenvironment (TME) to shield themselves from the immune system as part of the cancer immunoediting process. They do so by secreting and expressing a variety of immunosuppressive molecules that downregulate or inhibit the immune system, such as TGF-β, thus converting

macrophages from an antitumor state to a pro-tumor state. Macrophages in the protumorigenic state help the growth and propagation of tumor cells by inducing ECM cleavage, tissue remodeling, angiogenesis, chemoresistance, and tumor-associated macrophage recruitment. Mutations in cancer cells may also result in the exposure of different epitopes on the surface of the cells, thus equally modulating the immune system [36].

The most relevant tumor-related cells in the TME are tumor-associated macrophages (TAMs) and cancer associated fibroblasts (CAFs) or myofibroblasts, both of which aid in tumor progression. TAMs stimulate metastasis while myofibroblasts re-organize the ECM to facilitate metastasis and epithelial tumor cell migration.

2.4.6. TAMS

TAMs or macrophages infiltrating the tumor bed are found to stimulate angiogenesis as well as tumor development and progression. Macrophages represent the largest percentage of the immune cell population present in the TME. Ideally as a part of the immune system, the macrophages should eradicate tumor cells and halt progression when activated correctly. However, macrophages were found to be mostly pro-tumoral in the TME. Their presence in the TME was significantly related to poor prognosis in several types of cancer such as breast, ovarian cancers, and lymphomas [37]. These two opposing states, pro and anti-tumoral, can be classified as M1 and M2. M1 or classically activated macrophages are generally anti-tumorigenic, expressing pro-inflammatory cytokines such as TNF-α, IL-6, IL-12, and inducible NO synthase (iNOS). However, M2 or alternatively activated macrophages have increased expression of anti-inflammatory cytokines such as IL-10 and IL-1 decoy receptor, which inhibits T effector cells. This classification is however simplified. It has been shown that within each polarization, macrophage activation states are heterogeneous calling forth further subdivisions, each initiated by different regulators and having different functions. In the liver, additional phenotypes for macrophages have been identified such as the one associated with hepatocarcinoma. These TAMs exhibit mainly the M2 phenotype and promote angiogenesis by increased expression of VEGF, they also facilitate matrix remodeling to accommodate angiogenesis by producing matrix metalloproteases [38].

Tumor cells secrete colony stimulating factor 1 (CSF1), also known as macrophage colony-stimulating factor (M-CSF), which recruits CSF1 receptor-expressing macrophages to the TME [39]. TAMs secrete enzymes that help degrade the ECM as well as various growth factors such as EGF, which promotes EMT and invasiveness. They also express Chemokine (C-C motif) ligand 18 (CCL18), which stimulates the clustering of integrin in tumor cells thus providing an anchor to the ECM and facilitating invasion and tumor cell motility. Furthermore, TAMs secrete MMP, produce cysteine cathepsins and serine proteases, which contribute to the re-organization of the ECM to facilitate metastasis. For instance, collective cell invasion is promoted by MMP2 and MMP9 secretion from immature myeloid cells at the leading edge of the invading cluster in CRC.

2.4.7. CAFs

CAFs play an important role in the TME by secreting various cytokines such as IL-8, or growth factors such as VEGF, or MMPs or chemokines such as chemokine (C-X-C motif) ligand 12

(CXCL12) which drive tumor growth, neoangiogenesis and local invasion [34]. They were found to communicate with tumor cells via the CXCL12/CXCR4 axis, which promotes tumor migration. More importantly, CAFs rearrange the ECM forming channel-like structures through which cancer cells may grow and invade the surrounding tissue without having to undergo EMT, thus maintaining their characteristic epithelial features. In a study, CAFs were incubated with supernatants from CRC cells, which led to marked activation of the TGF-β pathway [40]. As seen previously, TGF-β is a known pro-tumoral factor inducing metastasis, which is also secreted from CAFs upon interaction with the TME. The secreted TGF-β in turn stimulates CAFs for further TGF-β secretion in an autocrine loop.

CAFs are also the primary source of ECM and connective tissue formation, such as collagens I, III, IV, V, and XII, and proteoglycans [41]. CAFs treated with TGF-β in vitro showed an overexpression of collagen type I, fibronectin, urokinase type plasminogen activator (u-PA), MMPs such as MMP-2 and MMP-9, and tissue inhibitors of metalloproteinases (TIMPs) [40]. On the genetic level, tenascin-C and laminin-B1 were also upregulated when compared with normal colon fibroblasts [41]. CAFs produce more MMPs when compared to CRC cells, thus making it the major player in ECM remodeling. Interestingly, collagen type IV, which is the main constituent of the basement membrane, is degraded by MMP-2 and MMP-9, which thus facilitate mesenchymal invasion [40].

Co-culturing of spheroid CRC cells and CAFs in a collagen invasion experiment showed that the spheroidal CRC cells in contact with CAFs had irregular edges with both individual cell and collective cell invasion of the matrix as opposed to the smooth-edged control CRC spheroids [42]. In another collagen invasion experiment, CRC cells treated with CAFs supernatants were found to have a five-fold increase in matrix invasion as opposed to control CRC cells [43]. Treated cells were also found to have a more elongated shape compared to the control CRC cells. CAFs were indeed shown to over-produce FGF-1, which activates FGFR-3, a receptor tyrosine kinase, leading to local invasion and cellular migration [44]. The pro-invasion morphological changes induced in CRC cells upon contact with CAFs supernatant confirm the multi-functional role they play in metastasis [45]. A recent study showed that CAFs were able to induce metastasis at a very early stage in tumor development, even when the tumor was of microscopic size, in a small number of cancer cells which remained associated with CAFs upon entering the circulation, potentially forming micrometastatic niches in distal organs which are only visible upon progression [46]. These mechanisms that induce tumor cell invasion and migration may be an interesting target for novel therapies in order to inhibit metastasis.

2.4.8. Role of the ECM

The ECM in particular may be defined as the collection of molecules surrounding the cells in a certain tissue, generally structural proteins such as collagens and elastins, enzymes such as metalloproteinases, polysaccharides, glycoproteins, water, signaling molecules and ECM bound growth factors. The ECM is vital for maintaining tissue homeostasis, proper scaffolding and structure. It is characteristic and unique for each type of tissue. It is a dynamic pool of molecules constantly edited by the surrounding cells in order to cater for their needs. Furthermore, ECM is an important motor for tissue growth, healing and cellular differentiation as demonstrated by studies in the developmental biology field. ECM is not only altered

by the type of cells present in the tissue but also by their state such as inflammation, injury, etc. Tumor beds in general share characteristic abnormalities in their ECM such as disorganized and disrupted ECM with extensive and uncontrolled neoangiogenesis. These changes in the ECM may drive the progression and invasiveness of tumors.

The colonic epithelium in particular is composed of polarized cells with a characteristic apical membrane, forming a barrier with the components of the colon lumen, a basal membrane attached to the basement membrane and lateral membranes attaching to adjacent cells. The basement membrane (BM) is a distinct structure of the ECM composed mainly of collagen type IV rich in disulfide bridges conferring the BM its rigidity, and of laminin, fibronectin and proteoglycans. Collagen type I replaces collagen type IV from the BM in the stromal ECM, which does not form disulfide bonds, making it less stiff than the BM [47].

Generally, tumors share common features with the ECM of unhealed wounds such as increased stiffness and epithelial contractility. They are characterized by dense growth in connective and fibrous tissues known as *desmoplasia* following injury or BM degradation [48]. Degradation of the BM is currently considered a marker for CRC and carcinoma progression in general. It has been associated with higher metastasis rates and worse prognosis as well as reduced patient survival [49]. Local tumor invasion is driven by two main consecutive mechanisms: enzymatic breakdown of the BM and migration of the malignant epithelial cells through the cleared ECM. A decrease in lateral cell–cell adhesion molecules like E-cadherin [50] is expected to allow for malignant cell detachment and migration. However, it has been shown that CRC may migrate as collective sheets [51]. Along with the enzymatic cleavage of the BM by MMPs, metastatic clones undergo cytoskeletal changes and form cell protrusions such as pseudopodes in order to migrate across the BM towards the mesenchyma, thus initiating tissue invasion [52]. BM degradation actively contributes to the progression of CRC since the ECM initially binds and presents growth factors and other modulators to surrounding cells. Disruption of the BM then releases these signaling molecules lodged within it such as angiogenic factors, growth factors and chemokines [53] thus advancing tumor growth, metastasis, neoangiogenesis and modulation of the immune system to a pro-tumoral state.

Laminin, a glycoprotein abundant in the BM, binds to integrin in epithelial cells, controls cell adhesion to the ECM, interacts with cell-surface receptors and adheres to other laminins thus giving the BM its strength [54]. Laminin-332 is ubiquitous and unique to the epithelium and was thus hypothesized to play a role in carcinoma development. Indeed laminin-332 cleavage products were found to activate the endothelial growth factor receptor (EGFR) pathway known to drive tumor proliferation, loss of cellular adhesion to the ECM and boosting migration [57]. It is a heterotrimeric structure composed of α3, a β3 and γ2 chains. The α3 chain, specifically its large globular domain 3 (LG3 domain) interacts with $\alpha 3\beta 1$ integrin thus enhancing cellular migration, adhesion and spreading on the ECM. Laminin-332 also interacts and binds to $\alpha 6\beta 4$ and $\alpha 6\beta 1$ integrins regulating actin-cytoskeletal protrusion, cellular migration and tissue invasion [55]. Degradation of the α3 chain resulting in cleaved LG3 and 4 domains were shown to be over-expressed in carcinomas which activate phosphoinositide 3-kinase (PI3K) and matrix metalloproteinases precipitating tumor growth and invasiveness. This was reversed in vivo with the use of antibodies against this domain of the α3 chain [56]. In colon and breast cancer, γ2 chain cleavage by membrane type-1 MMP was found

to stimulate cellular migration [57]. The migration-inducing effects of laminin-332 cleavage products contrast with that of intact laminin-332 itself, which promote epithelial cell adherence to the BM. These opposing effects are hypothesized to alternate under the effects of MMPs cleaving the laminin, thus maintaining tissue homeostasis. In CRC, MMPs are secreted by the tumor cells and surrounding inflammatory cells causing a shift in laminin activity to its cleaved form's migration and invasion inducing effects. All of these cleavage products expose a repeat of an EGF-like domain in the short arm of laminin-332 and become ligands to cell-surface EGFR, thus activating its proliferative and anti-apoptotic pathway [58].

In addition the non-collagenous proteins bone sialoprotein II and osteopontin have been found to be involved in the regulation of the ECM proteins MMP-7 and 9. The inverse regulation of Hoxc8, Runx2 implicates that these genes may be regulated in a feed-back loop manner [59, 60].

The formed dense collagen reorganized by the overexpressed LOX and the various metalloproteinases has a different orientation in CRC compared to normal healthy tissue. The collagen fibers become radially disposed at the interface between the epithelium and the stroma at an angle of 50°C as opposed to 10°C in the healthy colon. This structural change helps tumor cells migrate along the steeply aligned collagen fibers, thus aiding in local invasion of adenocarcinoma cells beyond the epithelium to the mesenchyma transformation [61]. High grade dysplasia was found to hoard changes in the ECM, harboring a denser and ordered collagenous fibers deposition, which reinforces the importance of ECM changes in malignancy, tumor progression and local invasion [62].

2.5. Intravasation

2.5.1. Mechanism

Intravasation is the process of invasion of the tumor cells into the blood or lymphatic vessels. In order for tumor cells to make contact with endothelial cells, complex interactions are needed with proteins lipids and carbohydrates. Carcinoma cells release various mediators and growth factors such as VEGF or vascular endothelial growth factor to promote the process of forming new blood vessels, also known as angiogenesis. The newly formed blood vessels have a leakier endothelial membrane than normal blood vessels, which may aid the process of intravasation and metastasis [63]. The lymphatic vessels are known to be formed of a mono-layer of endothelial cells without intercellular tight junctions. They also lack a basement membrane and smooth muscle cells to cover the endothelial cell layer unlike blood vessels making them an easy target for invasion [64]. Tumor cells are known to release VEGF-A, which is a potent activator of vascular endothelial receptor 1 (VEGF-R1) and R2, known to induce angiogenesis as well as the release of VEGF-C and –D, which activate VEGF-R3 known to induce lymphangiogenesis oriented towards the tumor cells. In turn, lymphatic vessels are thought to secrete chemokines such as CCL21, which might attract invading tumor cells [64].

It remains a topic for debate as to whether tumor cells migrate actively towards the blood and lymph vessels or whether it is a passive migratory process. The fact that the newly formed tumor blood vessels are immature, lacking organization and intercellular junctions may support the claim that tumor cells simply grow through the fragile endothelium, forming

clumps in the lumen due to intravascular proliferation [65]. However, strong evidence exists to support active migration, such as the change in tumor cell expression of growth factors and their receptors influenced by TME components such as TAMs and CAFs. Transient TGF-β activation of TGF-β type 2 receptor/Smad4 induced EMT and stimulated their invasion of blood vessels whereas prolonged TGF-β activation hindered the process [30]. In the lymphatic vessels, on the other hand, invading cells were found to be organized solely in non-EMT clusters and independent of TGF beta signaling [66]. It is therefore safe to assume that both active and passive mechanisms occur in the intravasation process of tumor cells, requiring both EMT and non-EMT cells for hematogenous intravasation (**Figure 2**).

2.5.2. Survival of tumor cell against circulating immune cells and sheering forces

In order for metastasis to be successful, disseminated tumor cells have to survive the immune system as well as the shearing forces of the circulation. It was shown that disseminating tumor cells are shielded by adherent platelets which protect the cancer cells from shearing forces and natural killer cells, as well as aid in extravasation [67]. Disseminating tumor cells were found to express membrane-bound tissue factor, which is activated by coagulation factors such as VIIa and X. Proteinase activated receptor 2 (PAR2) is activated by the Tf-VIIa complex causing immunomodulation, angiogenesis and evasion of apoptosis in the tumor cells [68]. Elevated platelet count and concentration was found to be correlated in a clinical setting with lower survival in colorectal, breast and lung cancers while treatment with anticoagulants lowered metastasis in cancer patients [69].

2.6. Extravasation

2.6.1. Mechanism

Once circulating tumor cells (CTCs) have found their way into the lumen of the blood vessels, they will extravasate and attempt to invade foreign tissues. Knowledge of CTC extravasation is modeled after leukocyte migration across the endothelium into target inflammatory tissue [35]. The extravasation process maybe both, active or passive. Organ specificity or tissue tropism of certain carcinomas may be due to a number of factors. The vascular structure may be more favorable in certain organs such as in the bone marrow, which has a single layer of endothelial cells thus facilitating the movement of red blood cells in and out of the bone marrow. This route constitutes an easy target for CTC extravasation, making the bone marrow a preferred destination for metastasis of various carcinomas such as breast, gastric and prostate cancers [70].

In the case of CRC, the organization and arrangement of the circulation is the major determinant of CRC metastasis. CRC is known to have a strong tropism for metastasis to the liver although the cells themselves may be poorly adapted to the liver environment. However, the portal circulation, draining directly from the mesentery into the liver, transports millions of tumor cells over from the colon to the liver microvasculature, making liver metastasis possible although otherwise it would have been highly unlikely. In these cases, extravasation and homing into a certain tissue is passive and dependent of the organization of the circulation

as well as its structure [5]. However, active processes are also at work where CTCs bind to specific features in the organ of interest such as TIMP1, which drives CRC metastasis to the liver by stimulating the HGF pathway [71]. We may thus conclude that extravasation at a certain site or homing of CTCs is dependent on mechanical and organizational features of the circulation as well as on specific interactions between the tumor cells and the endothelium in question (**Figure 3**).

2.6.2. Passive extravasation

Passive extravasation entails no active migration involving molecular interactions between CTCs and endothelial cells. The endothelium often sheds endothelial cells from its wall creating an opening through which CTCs enter into the parenchyma of the invaded tissue. The CTCs may also proliferate inside of the lumen which causes an increase in size and ends with the rupture of the vascular wall, giving full access to the target organ [72]. Furthermore CTCs are known to associate with platelets for protection within the circulation, therefore damage in the blood vessel wall, exposing fibrinogen on endothelial cells, attracts platelets and tumor cells alike. Fibrin clots can further damage the blood vessels, which attract even more platelets in CTC to the site of injury. Furthermore tumor cells may also migrate into other tissues following the migration pattern of white blood cells within the circulation.

2.6.3. Active extravasation

Countless studies have shown that homing and extravasation might be more than simply a mechanical response to a faulty environment. TAMs were shown to secrete TNF-α, VEGF and TGF-β into the circulation to distal tissues where they activate tissue macrophage production of S100A8, which is a chemoattractant for CTCs [46]. CTC attachment to the endothelium is allowed by endothelial cell P- and E-selectins binding to tumor cells as well as tumor glycan patterns and adhesion molecule interactions such as integrin and CD44 interactions [73]. Overexpression of mucin carbohydrate is correlated with increased metastasis in CRC. Constituents of the ECM such as laminin and fibronectin may increase the arrest of

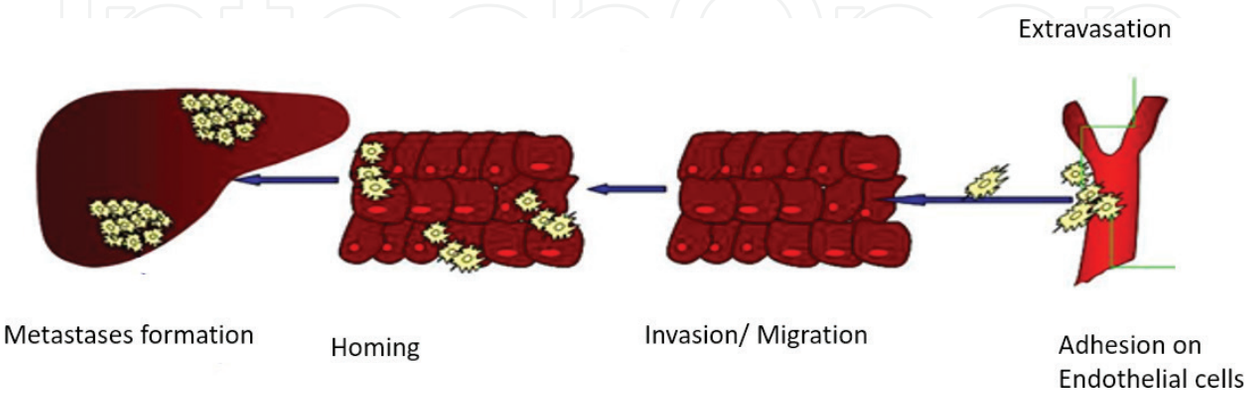

Figure 3. The tiny fraction of the disseminated tumor cells that were able to survive the previous steps must undergo extravasation, migration and homing into the distant organ with different histological nature. Adaption of the new environment demands further physiologic elasticity from the cells.

tumor cells at a certain site in the vasculature. However, when this interaction is counteracted by targeting peptides against fibronectin and laminin, it was found that it reduces the formation of metastasis. Circulating tumor cells may also increase vascular permeability and cause retraction of endothelial cells to expose the ECM for attachment by secreting VEGF which activates SRC (**Figure 3**).

2.6.4. Chemokines and colorectal cancer

Multiple interactions between tumor and stromal cells often contribute to the tumor progression. These interactions are mediated by a variety of growth factors, enzymes and chemokines. In this regard, chemokines play their role in three possible routes, which are enhanced proliferation by auto-or paracrine manner (a), modulating the immune response (b) or favoring the angiogenesis (c). Effects of chemokines in CRC are quite vital in terms of tumor growth and its metastasis. Many of the clinical studies have indicated the expressional changes of chemokines in CRC development and progression. CXC-chemokines are more important in this regard, as these chemokines have been shown to modulate the anti-tumor immune response, behavior of the epithelial cells and cross talk with the stroma. Chemokines are of vital importance in CRC metastasis, where chemotactic signals from different organs facilitate the directional migration of CRC cells, which lead to the metastatic state of the disease. In this context, several studies have highlighted the high expression of chemokine receptors (e.g. CXCR4, CXCR3, CCR6, CCR1 and CCRL2, CCR7 and CCR5) which favor the metastasis of CRC to lymph nodes and liver [47, 74, 75, 23].

2.7. Homing

For the immune response in host tissues, a focus is made on Kupffer cells in CRC liver metastasis. In the case of CRC metastasis to the liver, Kupffer cells (KC), which are the resident liver macrophages, have been reported to directly kill cancer cells through the secretion of cytotoxic molecules such as TNF-α, reactive oxygen species and enhancing the antitumor response of other immune cells such as T effector cells. They have also been reported to have a protumorigenic effect by producing signaling molecules such as cytokines and chemokines, which promote angiogenesis, ECM remodeling and cleavage and recruitment of TAMs. KC are the only characterized macrophages that exhibit dectin-2 receptor mediated cancer cell phagocytosis [76].

In a mouse model of colorectal cancer liver metastasis, KC were chemically depleted by gadolinium chloride before tumor induction and at a later stage of tumor growth, after liver colonization. Absence of KC at the early stage of liver metastasis and invasion led to an increase in tumor burden compared to mice with intact KC at the same stage of liver metastasis. Depletion of KC however in the later stages of liver colonization (18 days) decreased the tumor load with an increase in the number of activated cytotoxic T-cells (CD3+ T cells) and infiltrating cells expressing iNOS with a decrease in the number of VEGF-expressing infiltrating cells, as opposed to non-depleted animals. These results point towards a bimodal role of KC in liver metastasis and tumors, with an antitumor effect in early stages of metastasis, before tumor

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establishment in the liver, and a protumorigenic effect at later stages of liver colonization and metastasis. Deeper understanding of the precise contribution of KC in CRC liver metastasis may be beneficial for timing immunomodulatory therapies [77]. It was also observed that the occurrence of CRC liver metastasis is rare in patients with cirrhotic livers. Upon closer investigation, it was found that a rat colon cancer cell line (RCN-9) pretreated with conditioned media of KC from cirrhotic rat livers and then inoculated into rat liver showed a reduced incidence of hepatic colonization. In vitro, RCN-9 cells were found to be sensitized to TIL-FasR-mediated killing after treatment with cirrhotic KC media by upregulation of FasR on RCN-9 cells [78]. This further confirms the versatility and importance of the role KC may play in tumor progression.

3. Antimetastatic treatment for CRC

3.1. Targeted therapies for metastatic detachment

To effectively eliminate metastatic cancer cells, it is suggested that both anoikis-dependent and anoikis-independent pathways should be targeted. Fortunately, many of the signaling pathways are already the targets of current FDA-approved therapeutic drugs such as bevacizumab (Avastin) against VEGF, ramucirumab (Cyramza) against VEGF receptor, cetuximab (Erbitux) and panitumumab (Vectibix) against EGF receptor [6].

However, there are significant molecular differences between tumors, which can affect both prognosis and response to treatment. Personalized medicine aims to tailor treatment according to the characteristics of the individual patient and is now a clinical reality as testing for *KRAS* mutations to guide treatment with the anti-EGFR monoclonal antibodies cetuximab and panitumumab is now part of routine clinical practice. However, not all patients who are *KRAS* wild type respond to anti-EGFR therapy and a validated biomarker for antiangiogenic therapy is still lacking. Therefore, other molecular biomarkers are needed to assist with predicting response to both existing drugs as well as to drugs currently under investigation [79].

3.1.1. Role of personalized medicine in metastatic detachment

Advances in the treatment of metastatic colorectal cancer have led to an improvement in survival from 12 months with fluorouracil monotherapy to approximately 2 years. However, there are significant molecular differences between tumors which can affect both prognosis and response to treatment. Personalized medicine aims to tailor treatment according to the characteristics of the individual patient, specifically for early metastatic prognosis and biomarker precision application to personalized treatments. In metastatic colorectal cancer an improved understanding of the underlying pathology and molecular biology has successfully merged with advances in diagnostic techniques and local/systemic therapies as well as improvements in the functioning of multidisciplinary teams, to enable tailored treatment regimens and optimized outcomes. Indeed, as a result of these advancements, median survival for patients with mCRC is now in the range of 20–24 months, having approximately tripled in the last 20 years.

3.1.2. Anti-epidermal growth factor receptor therapies in mCRC personalized treatment

The first true use of personalized medicine in mCRC was the clinical testing of *KRAS* mutations (which occur in approximately 45–50% of patients with CRC) [80]. Subsequently the anti-EGFR treatment is given only to patients who are *KRAS* wild type. However, not all patients who are *KRAS* wild type respond to anti-EGFR therapy and therefore there has been substantial research into other potential predictive biomarkers for future precision application [81].

3.1.3. BRAF mutation in personalized therapy of mCRC

After KRAS mutations, BRAF V600E mutations currently have the strongest evidence to support their use as a predictive biomarker for EGFR-targeted mAb activity. Overall, BRAFV600E activating mutations occur in approximately 10–15% of CRC tumors and are generally mutually exclusive to KRAS mutations [82]. Most but not all of the available evidence links BRAF V600E mutations with resistance to EGFR-targeted mAb therapy [83], however, the impact of tumor BRAF status on efficacy of these treatments has not yet definitively been addressed due to the relatively small number of patients with BRAF mutations.

3.1.4. The PI3K pathway in mCRC personalized therapy

The main alterations in the PI3K pathway in CRC are mutations in PIK3CA and loss of PTEN protein expression. These molecular alterations may coexist with KRAS and BRAF mutations and this makes it more challenging to ascertain their clinical significance [84]. However, PTEN loss correlates with advanced and metastatic tumors and has been associated with worse survival outcomes in CRC.

Several studies revealed that PIK3CA mutations or PTEN loss are associated with a lack of response to anti-EGFR therapies and these alterations therefore appear to have a negative predictive role [85].

Moreover, the personalized strategy has yet developed to be exon specific. For example, mutations in exon 20 of PIK3CA have been associated with a low response rate to anti-EGFR therapy, whereas mutations in exon 9 do not appear to have this effect, which leads to taking research on mutation correlation with the metastatic therapy more specific [86].

Huge advances have already been made, which can be exemplified by recent progress in the management of mCRC, particularly the discovery and implementation of *KRAS* as a predictive biomarker. Indeed, the implementation of new technologies is leading to the accumulation of huge amounts of genomic and proteomic data and the identification and validation of predictive biomarkers for existing and new targeted therapies and will likely improve patient outcomes in the future.

True personalized medicine in mCRC currently remains an aspiration for the future rather than a clinical reality. However, it is likely that a molecular screening approach to treatment will become increasingly used in the future to fully characterize tumors and identify patients who are most likely to benefit from targeted treatments. This holds great promise for the

improvement of patient outcomes but brings its own logistical and financial challenges as well as new complexities, such as how to overcome tumor heterogeneity, how to interpret a patient's molecular profile to select the most appropriate treatment and how to prevent rapid development of treatment resistance.

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