

Extracellular Matrix and Colorectal Cancer: How Surrounding Microenvironment Affects Cancer Cell Behavior?

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Abstract

Colorectal cancer (CRC) with more than a million of new cases per year is one of the most common registered cancers worldwide with few treatment options especially for advanced and metastatic patients.

The tumor microenvironment is composed by extracellular matrix (ECM), cells and interstitial fluids. Among all these constituents, in the last years an increased interest around the ECM and its potential role in cancer tumorigenesis is arisen. During cancer progression the ECM structure and composition became disorganized, allowing cellular transformation and metastasis.

Up to now, the focus has mainly been on the characterization of CRC microenvironment analyzing separately structural ECM components or cell secretome modifications. A more extensive view that interconnects these aspects should be addressed. In this review, biochemical (secretome) and biomechanical (structure and architecture) changes of tumor microenvironment will be discussed, giving suggestions on how these changes can affect cancer cell behavior.

Introduction

Colorectal cancer (CRC) is the third most common cancer in both sex with more than a million of new cases per year and more than 500.000 deaths registered worldwide [1]. If diagnosed at early stage (stages 0, I, II, and III), CRC is in general highly treatable with surgery. For patients with stage III and some with stage II, the chemotherapy is practiced after surgery in order to increase the therapeutic response [2]. In the clinical practice, the established staging models for CRC such as the TNM [3] and the tumor localization are based on morphological, histopathological and clinical criteria, and should provide adequate information on the tumor metastatic potential directing clinicians to plan an adequate therapeutic strategy. Possible new markers may result from the study of extracellular matrix (ECM) components, particularly those that provide the evidence of aggressiveness. These new markers could ameliorate the actual clinical staging differentiating patients with an aggressive CRC from those with more indolent disease.

In the last decades, an increased interest around the ECM and its potential role in cancer tumorigenesis is arisen [4-5]. The ECM, with its distinctive biochemical and biomechanical properties, is one of the proximal structures that tumor cells of epithelial origin must destroy in order to permit invasion and cell migration. The ECM structure and composition became disorganized during cancer progression, promoting itself cellular transformation and metastasis. Indeed the ECM microenvironment, or niche, plays an important role in regulating cell behavior. This concept was initially true in the field of developmental biology, and in the past 20 years was also demonstrated for cancer development and biology [6-7]. Several components of the niche participate to cancer progression, especially inflammatory cells, such as T cells and myeloid suppressor cells, which fail to exercise antitumor effector functions, and co-operate with cancer cells to promote tumor growth [8]. Together with cellular component, recent progress highlighted the ECM importance in cancer progression [9-12]. During embryonic development and organ homeostasis, ECM is tightly controlled with the production, degradation, and remodeling of the major protein components. The disruption of this fine control disorganizes the ECM, promoting abnormal cell behavior, tumor-associated angiogenesis and inflammation, and finally leads to generation and progression of cancer microenvironment. In this review, we describe how biochemical and biomechanical changes could drive tumor progression and aggressiveness, with suggestions on how these changes could be chosen as therapeutic targets for cancer treatment.

The colon ECM structure

The normal colon ECM structure is reported in Figure 1. The regular and organized structure keeps under control cellular processes such as growth, death, adhesion, migration, and differentiation. Its function is to maintain homeostasis and to orchestrate tissue repair in case of injury or damage.

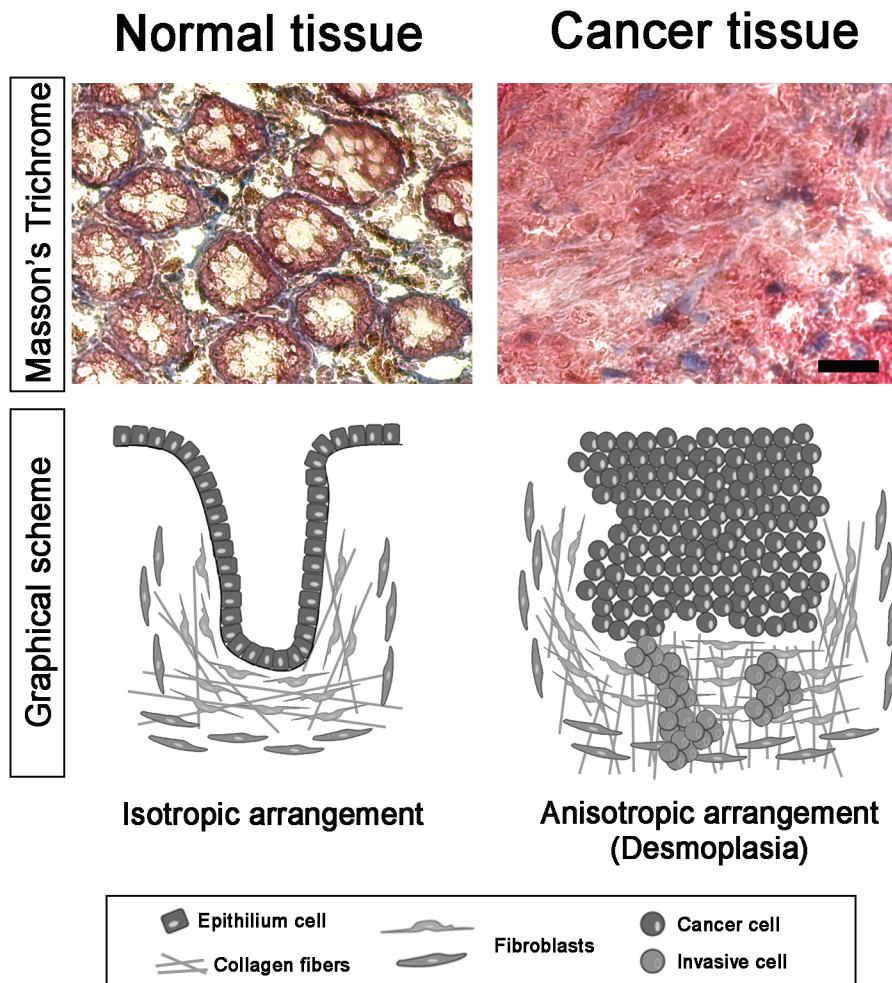


Figure 1. Schematic representation of normal and colon cancer tissue. Upper panel: Masson's Trichrome staining showing the connective tissue (blue), nuclei (dark red/purple), and cytoplasm (red/pink). Lower panel: graphical scheme highlighting the transition from the normal colon (Isotropic) mucosa to the cancer (anisotropic) microenvironment.

Colonic epithelial cells exert a physical barrier with absorptive and exocrine functions. These cells possess a polarized structure in which is recognizable an apical pole, two lateral surfaces forming inter-cellular connections and a basal surface anchored to the basement membrane (BM). The BM is a specialized form of ECM separating the colon mucosa from its submucosa. The BM proteins

are synthesized by both the epithelial and the mesenchymal cells under physiological conditions [13-14] and rearranged to a characteristic sheet-like structure of 100-300 nm of thickness. The main constituent of BM is the Collagen IV, a network-forming collagen composed by a trimerization of alpha chains (two α 1- and one α 2-chain) encoded by six genes (*COL4A1-COL4A6*) and other proteins such as the proteoglycan perlecan and the glycoproteins laminin, fibronectin and nidogen [15]. From a structural point of view, the stromal ECM is constituted by similar components already present in the BM, but Collagen IV is substituted by Collagen I (*COL1A1, COL1A2*), this last forming a 3D structure thanks to its fibrillar properties. The Collagen I is produced by resident fibroblasts and, differently from Collagen IV, no disulphide bridge are present, thus providing a less rigid structure to the ECM with respect the BM. For several years the ECM was considered a solely filling and inert substrate, but now its active role in carcinogenesis is clear [16-17].

Beside the structural proteins, other constitutive elements of ECM are those proteins and molecules secreted by tumor and stroma cells and “collected” under the term *secretome*. The classical way of secretion is based on soluble proteins released after the signal peptide cleavage, however also non-classical ways have been identified in tumor secretome including exosomes [18-19] and microvesicles secretion [20]. Recently, Naba *et al.* [21] introduced the term *matrisome* to gather all ECM components by the *in silico* prediction of putative ECM proteins. Further proteomics data on extracted ECM samples confirmed their classification. Under this term, more than one hundred of putative ECM proteins have been categorized in two distinct groups: *i*) structural core matrisome and *ii*) matrisome-associated proteins including secreted factors, proteases and protein families such as mucins, galectins and semaphorins [21].

Changes in ECM structure in colorectal cancer

The tumor mass in CRC, like every other tumor, has three basic components: *i*) the parenchyma, i.e. the proliferating neoplastic cells and the supportive stroma *ii*) the tumor associated cells (TAM, tumor associated macrophages and the activated fibroblasts) and *iii*) the blood vessels.

As mentioned before, under physiological and non-pathological conditions the ECM, composed of BM and adjacent parenchyma, possesses isotropic arrangement of fibrillar protein components. An orderly ECM confers to the matrix unique spatial, biochemical and biomechanical properties that are essential for regulating cell behavior and tissue homeostasis. On the contrary,

an organized, anisotropic arrangement of ECM fibers is a recognized hallmark of a pathological microenvironment [22-23] and the term desmoplasia is used to define the abundant collagenous stroma surrounding parenchymal cells that is deposited after BM degradation. It is important to underline that the BM degradation is not only a passive event, but actively participate to the tumoral progression releasing angiogenic, growth-stimulating, and chemotactic factors embedded within the BM [24-26]. All these factors affect the tumor microenvironment, regulating tumor growth, angiogenesis, and cell migration. This is the case of laminin-332 (formerly known as laminin-5) protein, which is homogeneously present in the BM in the normal intestinal mucosa. The laminin-332 degradation products activate the EGF receptor pathway causing a diminished cell-matrix adhesion, ultimately leading the migration enhancement [27-29]. The loss of BM integrity is one of the most common ECM marker studied up-to now and recently reviewed by Mylonas *et al.* [30], who pointed out that in primary CRC tumors the discontinuity of the BM is correlated to a higher metastatic potential [31] and poor patients' survival rate [32].

The newly deposited collagen replace the proteolitically degraded ECM proteins by secreted proteases. In the case of breast cancer, this structural evolution is known as *tumor-associated collagen signatures* (TACS). Three levels have been identified denoting changes in fibers organization during the tumoral progression from nonpalpable (TACS 1) to non-invasive (TACS 2) and metastasis (TACS 3) [22]. Such a change causes local cell migration that is predominantly oriented along radial aligned collagen fibers, facilitating the tumoral invasion. Recently, it has been demonstrated that a similar event occurs also in CRC: in the healthy colon, collagen fibers are disposed in the epithelium-stroma interface whit an angle of $\sim 10^\circ$, while in the adenocarcinoma collagen fibers are thicker and showed an averaged angled disposition around the 50° with respect to interface [33]. In another work, changes in collagen density and alignment have been demonstrated in polyps with high-grade dysplasia (HGD) and malignant samples. With respect to the normal colon tissue, in which are present aligned collagen fibers, the HGD revealed the presence of misaligned collagen fibers. The resultant changes in the ECM of malignant samples is a more dense and ordered collagen fiber deposition [34] suggesting a pivotal role of collagen in malignant tissue transformation.

Collagen degradation during stroma remodeling releases several fragmented peptides which can be easily detected in plasma and urines [35] and two peculiar peptides of type I Collagen are indicative of the degradation process: the N- and C-telopeptides. Beside these, other fragments from the N- and the C-terminal regions, the PINP (the amino N-terminal propeptide)

and the PICP (the carboxy C-terminal propeptide) are indicative of collagen neo-synthesis [36]. Both the PICP and the C-telopeptide levels in serum of CRC patients (stages I-IV) have been found increased with respect to the control group [37], indicating the presence of both collagen degradation (tumoral mass expansion) and deposition (ordered fiber deposition). As a consequence of ECM remodeling, triple-helical region fragments have been detected and quantified in urine of patients affected by CRC with a sensitivity of 88% and a specificity of 88% [35].

The liver is the most frequent site of metastasis from primary colorectal cancer [38]. With difference to colon mucosa, the liver is enriched in type IV Collagen fibers mainly located in the sinusoids and, together with perlecan and laminin is the main constituent of organized BM [39]. It is known that Collagen IV plasma levels are generally increased in liver diseases associated with hepatic fibrogenesis and fibrosis [40]. Because of a similar remodeling process occurs both in primary cancer and in distant metastasis, the Collagen IV can be used as prognostic marker of disease progression [41-42]. As a proof of this, in the plasma of patients presenting hepatic metastasis, type IV Collagen increases compared to non-metastatic CRC patients and healthy controls. Moreover, the ECM remodeling process combined with the increased expression of the $\alpha 1$ - and $\alpha 2$ - chains of type IV Collagen has been observed in the stroma of hepatic metastasis [43]. A deeper observation evidenced that type IV Collagen plasma levels were also correlated to the therapeutic response and pathology progression showing a decrease during chemotherapy and an increase in consequence of disease progression [43]. The prognostic value of hepatic Collagen IV is increased by the concomitant detection of other ECM proteins such as tenascin-C, fibronectin, and laminin. This composition has been highlighted to be peculiar of 'non-capsular' tumors and their detection, as well as the presence of alpha-smooth muscle actin positive myofibroblasts, was associated with a shorter median survival with respect to patients with "capsular" metastasis [44].

Beside collagens, other ECM proteins are deregulated in CRC cancer. Evidence of proteins alteration can be obtained by the comparative analysis of colon-purified matrices in normal and tumoral tissues in order to find other specific ECM signatures associable to cancer progression [45-46]. The technical procedure used to obtain ECM purified proteins was based on the assumption of their insolubility in traditional buffers, thus offering the advantage of sequentially separate and remove cellular and nuclear proteins from that of ECM. Despite this consideration, the major part of the identified proteins (1062 proteins) still were from cellular compartment and relatively few proteins were associated with ECM (163 proteins) [45]. Nevertheless, the paired

analysis of three primary metastatic colon cancers and their hepatic metastasis proved that 23 ECM-related proteins were in common for both tissues. Beside these, 37 and 7 proteins were exclusively present in the primary tumor and the metastasis, respectively. Tumor-derived proteins were mainly structural proteins such as ECM glycoproteins, collagens, and proteoglycans and other less abundant ECM-related proteins such as ECM remodeling enzymes and ECM-associated secreted factors. As expected, proteases were peculiar of primary colon tumor: ADAM 9, 10, TSL1 and MMP1, 2, 9, 11, and 12 have been found solely in colon tumor [45] and not in the metastasis, suggesting their role in the migration process. In another paper, the paired biopsies from tumor and its normal counterpart were obtained from 13 patients. Fifty-six differentially expressed proteins have been identified in the insoluble tissue fraction, after the extraction of lipids and soluble proteins. The digested peptides from ECM fraction were analyzed using a nano-ESI source by means of label-free quantitation approach (e.g. solely based on measurements of observed peptide ion peak intensities). The obtained data highlighted that 31 proteins related to cellular cytoskeletal filaments were up-regulated (fold change >1.2), and 25 proteins related to ECM were down-regulated (fold change < 0.8). The down-regulated proteins included myosin, keratin, and collagen; in particular the three chains ($\alpha 1$, $\alpha 2$ and $\alpha 3$) of type VI Collagen were among the lower abundant proteins in cancer tissues [46].

Changes in ECM architecture in colorectal cancer

Directly linked with the above mentioned changes of biochemical properties, another outstanding characteristic of the ECM is its elasticity, that ranges from soft and compliant to stiff and rigid, and contributes to the development of disease. Increased matrix stiffness is typical of most solid tumors and these changes can be due to different mechanism. For example, excess activities of lysyl oxidase (LOX), an ECM-modify enzyme that among all ECM proteins cross-links especially collagen fibers, could increase tissue stiffness. Up-regulation of *LOX* expression has been found in several cancers, including CRC, and has been associated with poor prognosis [47-48]. The LOX-mediated collagen cross-linking, in *in vitro* and *in vivo* models of CRC results in increased tissue stiffness and activation of the FAK/SRC signaling. As a consequence, cells expressing high levels of enzymatically active LOX protein have an increased capacity to proliferate, invade and metastasize [49], data that support the correlation between stiffer stroma and cancer aggressiveness.

When ECM became stiff, with formation of cross-linked collagen bundles, its biomechanical properties change, and cells respond by exerting markedly different kinds of attitude, as demonstrated by recent studies on mechanotransduction [50]. Balancing cell proliferation and apoptosis is essential to proper tissue function. A disruption in this equilibrium can result in tumorigenesis if there is inadequate apoptosis coupled to uncontrolled cell proliferation. The Hippo pathway, which has been uncovered for the first time in *Drosophila* genetic screens, is a potent regulator of tissue homeostasis by controlling cell growth, division, and apoptosis. Recently, two fundamental Hippo pathway's elements, the Yes-associated protein (YAP) and its paralogue TAZ, have been shown to be directly involved in the mechanisms of response to structural changes in the cell microenvironment [51-52]. Mechanical stimuli, such as increase substrate stiffness, can trigger nuclear translocation of YAP/TAZ, initiating their interaction with other transcription factors to control cell proliferation and motility [53]. In cancer environment, YAP and TAZ over-expression induces cell proliferation and epithelial to mesenchymal transition (EMT), and reduces apoptosis and contact inhibition [54-55]. YAP is associated with clear cell ovarian tumors, an ovarian malignancy subtype with poor prognosis [56], and has been shown to play an oncogenic role in esophageal squamous cell carcinoma [57]. TAZ is directly involved in the progression of breast [58-59] and non-small cell lung cancer [60]. There are evidences that YAP and TAZ play an important role also in colon cancer: YAP has been found over expressed in 68 of 71 human colon cancer analyzed biopsies and in 30 of 36 colon cancer-derived cell lines, suggesting that this protein is likely to be an important driver in this malignancy [61].

Despite recent advances in the field, it is not clear yet if changes in stiffness can play a causative role in cancer pathogenesis. In breast cancer has been demonstrated that ECM deregulation is one of the primary inducers of tumorigenesis [11], suggesting that this type of mechanism can be implicated in tumor initiation also in other tissues.

Changes in colorectal cancer secretome

Secreted proteins play important roles in homeostasis, immune response, development, proteolysis, adhesion, and ECM organization. Moreover, in a living organism secretome is highly dynamic, and its composition changes during cell differentiation [62] or in response to various pathologies [63] and/or environmental stimuli [64-65]. In the last decades, the tumor secretome has been judged a promising source for biomarker discovery and it has been extensively investigated [66]. The tumor microenvironment is mainly composed by cells, ECM and interstitial

fluid (IF); this last is enriched in tumor-derived proteins secreted by classical (e.g. ER/Golgi apparatus mediated) or non-classical (endosomal recycling, active protein transport, membrane translocation and exosomes) pathways [67].

It has been largely demonstrated that not only the tumoral cells, but the cancer-associated fibroblasts (CAF), the inflammatory cells (e.g., TAM) and the endothelial cells can also actively participating to the secretome composition in the tumor microenvironment. This causes a differential expression of proteases and cytokines in tumor cells compared with tumor-associated stroma [68-69]. During tumor invasion and metastasis, tumor cells directly secrete degradative enzymes or induce the host to produce proteolytic enzymes to degrade ECM. Proteolytic enzymes are major players in the breakdown and reconstruction of ECM in a wide of physiological and pathological processes. So great attention is paid on the aberrant expression and the predictive value of the proteolytic enzymes (and their inhibitors), such as: MMPs [70], plasminogen activation system [71], ADAMTSs [72] and cathepsins [73]. Table 1 summarized the changes in expression and proteins levels of these proteolytic enzymes observed in CRC. In particular, in CRC two key gelatinases (MMP-2 and MMP-9) and two key collagenases (MMP-1 and MMP-13) have been extensively investigated and a good review on this topic is already present [74]. The CAF produce and secrete growth factors and cytokines that promote tumor survival [75] and aggressiveness [76], while the recruited inflammatory cells secrete angiogenic growth factors, cytokines and other MMPs [77-78], which ensure the neoplastic progression and invasion. For instance, it has been demonstrated that the MMP-2 is mostly synthesized by the inflammatory infiltrated cells and only in a less extent by the tumoral cells itself [67]. In addition, endothelial cells can be activated by tumoral cells. The release of hypoxia-inducible factors (HIF) and vascular endothelial growth factor-A (VEGF-A) [79] by cancer cells stimulate the autocrine secretion of VEGF and angiopoietin [79-80] in endothelial cells, with consequent tumor angiogenesis and vasculogenesis.

Table 1: Overview of secreted proteolytic enzymes and their physiological inhibitors involved in the colorectal cancer (CRC). Legend: ADAMTS, a-disintegrin and metalloproteinase with thrombospondin motifs; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; uPA, urokinase-type Plasminogen Activator; tPA, tissue Plasminogen Activator; PAI, Plasminogen Activator Inhibitor.

Recommended name:	Alternative name	Changes in protein levels/gene expression	Ref.
ADAMTS-15	-	Decreased expression in colon cancer	[81-83]
ADAMTS-18	-	Decreased expression inactivated in colon cancer	[83-84]

Cathepsin B	APP secretase	Increased tumor tissue levels	[85-88]
Cathepsin L1	Cathepsin L	Increased tumor tissue levels	[87, 89]
MMP-1	Collagenase-1	Increased plasma levels and tumor tissue expression	[70, 90-91]
MMP-2	Gelatinase A	Increased tissue levels in tumor and metastasis Decreased serum levels in 72 CRC patients	[70, 92] [93]
MMP-7	Matrilysin	Increased expression and protein levels in liver metastases samples	[94-96]
MMP-9	Gelatinase B	Increased tissue and serum levels in tumor and liver metastasis	[92, 97-99]
MMP-12	Metalloelastase	Increased tissue levels in metastasis	[68, 100-101]
MMP-13	Collagenase-3	Increased tumor tissue levels	[102-105]
PAI-1	Serpin E1	Increased expression in stroma cells at tumor tissue levels or in co-cultures Higher plasma levels	[69, 106-108] [109-110]
PAI-2	Serpin B2	Increased expression at tumor tissue level	[110-111]
TIMP-1		Increased plasma and tumor tissue levels in CRC patients	[112-114]
TIMP-2		Decreased serum and tissue levels	[93, 115]
TIMP-3		Decreased tissue levels in CRC patients	[116-118]
TIMP-4		Increased levels in rectal cancer patients predict longer survival	[119]
uPA		Increased tissue levels in colon and rectal cancer	[71, 109, 111]
tPA		Decreased tissue levels and activity in CRC patients	[120-122]

The secretome is an important source of biomarker discovery, since the IFs are relatively enriched in proteins otherwise not easily detectable at blood or plasma levels. As a proof of this, some authors provided the match of cell/tissue secretome with plasma levels of detected proteins and positively tested their clinical value as biomarkers in CRC [123-124]. Mass spectrometry-based proteomic approaches are the best way to investigate the secretome, since this technology leads to detect hundreds of potentially secreted proteins per analysis. To identify with confidence such a number of proteins, both pre-analytical and analytical issues are of fundamental importance. For instance, sample pre-fractionation before mass spectrometry analysis by means of gel electrophoresis (i.e. 1/2-DIGE or off-gel separations) [125] or proteins enrichment procedures [126-127] are usually performed to diminishing the ion suppression phenomena or to increase the

low-abundance proteins concentration, respectively. Together with nano-ESI sources and high sensitive instrumentation, more than 2000 unique proteins have been identified in a single colon secretome sample [128]. Curiously, the final step of this workflow requires the reduction of data complexity by means of statistics [123] and bioinformatics [129], in order to pick-up few, but extremely significant proteins.

The analysis of secretome changes during the development and progression of CRC has been performed using different approaches. In Table 2, the comparison of biological source of secretome, the experimental procedures and the most interesting results obtained are reported. In the simplest protocols, cancer cell lines are cultured in traditional medium until confluence, then cultures are shifted into a serum-free medium for at least 20h, in order to reduce the contamination of non-endogenous proteins. Despite this approach minimize the sample complexity, the collected conditioned medium (CM) is an extreme simplification of what really occurs *in vivo*, not including all the other cells that participate to the secretome composition. Indeed, the co-cultures of colon cancer cells with CAF or TAM highlighted the dramatic changes in their secretome composition with respect to cancer cell cultured alone [78]. In fact, Kang *et al.* demonstrated in different co-culture of colon cancer cells lines (HCT116, WiDr, SW480, and RKO) and TAM cell lines (THP-1 and U937) that the latter cells cause the up-regulation of tumor cell-derived MMP-2 and MMP-9, with increased tumoral invasiveness and migration with respect to single cultured cancer cell line [78].

Table 2: Overview of secretome studies in colorectal cancer in cells and tissues performed using mass spectrometry-based proteomic approach.

cell tumor cultures				
Source	Procedure	Evidence	Ref.	
Culture of HT29 human CER cells.	Microvesicles collection by ultracentrifugation; LC-MS/MS analysis.	Identification of proteins involved in MV formation and tumorigenesis.	[125]]
Culture of LIM1215 cell lines and xenograft mouse model.	Culture in 1% ITS, 24h; xenograft minced in PBS, 1h; LC-MS/MS analysis.	39 glycoproteins identified, among those CDH17, LGALS3BP, and PTK7 were found both <i>in vitro</i> and in the interstitial fluids.	[126]]
Paired tumoral cells of primary CRC and lymph node metastasis.	Serum-free CM, collected in 20h; LC-MS/MS analysis.	Two candidate proteins (TFF3, GDF15) selected as biomarkers for the prediction of CRC metastasis.	[123]]
Culture of Colo205 and SW480 cell lines.	Serum-free CM, collected in 24h: MALDI-TOF/TOF.	CRMP-2 was found in CRC secretome; plasma levels were significantly higher in CRC patients.	[124]]
Culture of Colo205, SW620 and SW480 cell lines.	Serum-free CM, collected in 24h: MALDI-TOF/TOF and LC-MS/MS analysis.	109 unique proteins detected in CRC cell lines secretome.	[130]]
Culture of KM12SM and KM12C colorectal cancer cells	CM of heavy and light labeled cultures, 24 h. LC-MS/MS	155 proteins showed >1.5-fold change between the two cell lines, of them 3 have been validated	[131]]

lines. analysis. in serum: GDF15, S100A8/A9, and SERPINI1.

cell tumor and stroma cell co-cultures

Co-culture of colon cancer cells and CAFs.	Co-culture in serum-free media, 2 days; LC-MS/MS analysis.	Collagen type XII selected as dedifferentiation marker of myofibroblasts and/or cancer cells.	[132]
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tissue secretome

Paired primary colon cancer and liver metastasis vs. normal tissues.	ECM decellularization by sequential extractions; LC-MS/MS analysis.	ADAMs and MMPs found solely in primary colon tumor; TIMP1 secreted by both primary cancer and liver metastasis.	[45]
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Paired plasma and xenograft-derived secretome; comparison with cell line secretome.	Interstitial fluids collected in PBS, 1h; glycoproteins enrichment and LC-MS/MS analysis.	39 glycoproteins were identified in cell lines secretome, five of them were confirmed in xenograft model.	[126]
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Paired CRC (stages I,II) and normal colorectal tissues from 9 patients.	Pieces of 1-3mm ³ in serum-free CM, 24h; lectin-based enrichment; LC-MS/MS analysis.	Among the differentially secreted proteins there are several proteins from fibulin, serpine and peptidase S1 families.	[127]
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Paired tumor and normal colon mucosa from 4 patients.	Pieces of 1mm ³ in PBS, 1h; LC-MS/MS analysis.	Selection of 76 proteins showing five-fold changes in cancer tissues secretome.	[128]
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Although it is known that few hours of starvation can affect protein expression and phosphorylation, the use of stable isotope labeling techniques has been suggested to distinguish newly synthesized proteins from those already present in standard serum-media [133]. The CM from cancer cell lines has been investigated by several authors [125-126, 130]. The secretome characterization of two CRC metastatic cell line models, the highly metastatic KM12SM and the poorly metastatic KM12C cell lines, has been obtained using a quantitative stable isotope labeling (SILAC) approach of conditioned serum-free medium [131]. Of the 155 differentially secreted proteins, the majority was implied in cell adhesion, migration, and invasion processes [131]. In particular, the authors correlated Neogenin (NEO1), Neuroserpin (SERPINI1), and Podocalyxin (PODXL) deregulation with cellular adhesion, while other secreted proteins SOSTDC1, CTSS, EFNA3, CD137L/TNFSF9, ZG16B, and Midkine with migration and invasion of highly metastatic cells. Finally, starting from these results the authors showed the efficacy of their proteomic approach to identify deregulated proteins that could be detected in human serum as new biomarkers for CRC. They evaluated the presence of growth/differentiation factor 15 (GDF15), Protein S100-A8 (Calgranulin-A), and Neuroserpin (SERPINI1) in the serum of 40 CRC patients and 20 controls: increased levels of these proteins were detected in cancer patients with respect to control group. The biomarker validity in terms of clinical application has been further evaluated by multivariate ROC curves of these three proteins. The Area Under the Curve (AUC) of ROC curve revealed that

these biomarkers were moderately accurate (AUC=0.884) in the discrimination of patients with CRC and controls with a discrete sensitivity (60%), but a high specificity (95%) [131].

Tissue secretome is also an important biomarker source. From a proteomic-based analysis, two different approaches are proposed in literature: the first recovered the tissue secretome by protein passive diffusion from the tissue biopsies into PBS solution [126-128]. In the second, the tissue is subjected to a sequential extraction procedure and the ECM scaffold is finally totally digested before the mass spectrometry analysis [45].

As an example of the first-cited protocol, the label-free quantitative proteomics approach has been used to find secreted biomarkers useful for the detection of lymph node metastasis [123] in paired normal/tumor colon tissue of 4 patients. More than one hundred of candidate biomarkers were obtained by the analysis of differentially abundant proteins (>1.5-fold change). Among these, two peculiar proteins have been selected and quantified in serum samples of a larger cohort of patients, the trefoil factor 3 (TFF3) and the GDF15.

The second protocol has been proposed by Naba *et al.* The paired analysis of metastatic CRC and its hepatic metastasis has been performed [45] using an ECM enrichment procedure based on the cells lysis and sequential extraction of intracellular components. This procedure highlighted some differences in the secreted proteins and, in particular, most of ECM-remodeling enzymes such as: the ADAM 9, 10 and the MMP-1, 2, 9, 11, 12 and LOXL1. These molecules have been identified exclusively in the primary colon tumor secretome. Despite these promising findings, it is still to investigate the impact of the sequential extraction procedure on the possible loss of low molecular weight secreted factors, such as chemokines, growth factors and cytokines. For instance, among the detected proteins the lack of interleukin-7, which is known to be secreted by colorectal cancer cells to promote the expansion of tumor infiltrating lymphocytes [134], could be ascribed to the aggressiveness of the sequential extraction procedure.

Conclusions and future perspectives

The idea that changes in ECM architecture may allow cancer development and progression is quite new, and the possibility that these changes could be used as diagnostic or therapeutic tools in cancer treatment is still poorly investigated. For instance, the desmoplastic reaction characterized by Collagen I deposition and altered Collagen IV distribution can directly affects the tumor response to treatment. This change in collagen composition has direct influence on the

tumor cell survival and resistance to 5-fluorouracil (5-FU), camptothecin and etoposide [135-136]. Conti *et al.* demonstrated in 3 human CRC cell lines (HT-29, KM12SM and KM12c) that cells cultured in presence of wild type Collagen IV and newly deposited fibrillar Collagen I differently react to therapy. A significant alteration in apoptosis in response to 5-FU has been effectively observed in cultures on fibrillar collagen compared to Collagen IV, and this change has been related to an integrin-mediated mechanism [135]. Moreover, the earlier study of Kouniavsky *et al.* [136] revealed that tumoral cells cultured onto both single protein layers and ECM-derived layers significantly change their response to chemotherapy. The stroma-derived ECM, and in particular the fibroblast-derived ECM, rendered the LiM6 cell lines more sensitive to the 5-FU. On the contrary, the same fibroblast-derived ECM showed a protective effect on LiM6 cell lines against the etoposide-induced apoptosis. The colon cell lines cultured on fibroblast ECM-layers showed increased expression of two key anti-apoptotic proteins: the bcl-2 and the bcl-x(L). Nevertheless, a direct correlation between the ECM components (are they structural or secreted protein) and bcl-2 and bcl-x(L) expression is still lacking [136]. These data suggest a more complex role of ECM and probably the changes in terms of tissue stiffness and 3D organization take part of this process: the tumor stroma is typically stiffer than normal stroma, depending indeed on the substitution of physiological ECM components with other proteins that dramatically change the tumor microenvironment. For example, in the breast cancer, affected tissues can be 10 times stiffer than normal tissues [11, 137]. In CRC, the stiffness changes and its implication of YAP and TAZ in tumor progression was yet demonstrated [138], but only TAZ has been found to be a prognostic marker after a screening of YAP, TAZ and two downstream target genes in two independent CRC patient cohorts comprising 522 patients [139].

In conclusion, tumor stroma could appear to be a good candidate target for cancer treatment, since it promotes cancer progression, growth and aggressiveness. However, some recent studies demonstrated that inhibition of the stroma reorganization and alignment through drugs or genetic engineering, accelerates tumor growth and decrease survival [140-141]. This suggests that stroma possess a more complex role in cancer homeostasis and progression. For this reason, it will be of paramount importance to analyze in depth the role of cancer matrix composition and architecture to elucidate the mechanisms by which an aligned and stiff ECM can modulate tumor progression and aggressiveness. Once a better understanding of these mechanisms will be gained, new studies should be addressed to find, among the involved proteins

and pathways, new targets suitable for cancer treatment beside to helpful markers predictive for the patients' response to therapy.

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