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MicroRNA Dysregulation in Colon Cancer Microenvironment Interactions: The Importance of Small Things in Metastases

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Abstract The influence of the microenvironment through the various steps of cancer progression is signed by different cytokines and growth factors, that could directly affect cell proliferation and survival, either in cancer and stromal cells. In colon cancer progression, the cooperation between hypoxia, IL-6 and VEGF-A165 could regulate the DNA repair capacity of the cell, whose impairment is the first step of colon cancer development. This cooperation redirects the activity of proteins involved in the metabolic shift and cell death, affecting the cell fate. The pathways triggered by micro environmental factors could modulate cancer-related gene transcription, affecting also small non coding mRNA, microRNAs. MicroRNAs have emerged as key post-transcriptional regulators of gene expression, directly involved in human cancers. The present review will focus first on the intertwined connection between cancer microenvironment and aberrant expression of microRNAs which contribute to carcinogenesis. In particular, the epigenetic mechanisms triggered by tissue microenvironment will be discussed, in view of the recent identification of miRNAs able to directly or indirectly modulate the epigenetic machinery (epi-miRNAs) and that are involved in the epithelial to mesenchimal transition and metastases development.

Keywords Colon cancer metastasis · miRNA · Epigenetic regulation · Ku70/80 · CPTIA · IL-6 · VEGF-A165

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Abbreviations

CPT1	Carnitine palmitoyl transferase 1
CRC	Colorectal cancer
DNA DSBs	DNA double strand breaks
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FASN	Fatty acids synthase
HDAC	Histone deacetylase
HIF-1	Hypoxia inducible factor-1
HR	Homologous recombination
IL-1	Interleukin-1
IL-6	Interleukin-6
miRNAs	Micro RNAs
NHEJ	Non homologous end joining
s-CLU	Secreted clusterin
sIL-6R	IL-6 soluble receptor
TAMs	Tumor associated macrophages
TGF-β	Transforming growth factor-beta
TIMP-1	Tissue inhibitor of metalloproteinase 1
VEGF	Vascular endothelia growth factor

Introduction

Colon cancer develops as a result of the pathologic transformation of normal colonic epithelium to adenomatous polyp, which ultimately leads to invasive cancer [1–3]. Tumor induction and progression are characterized by accumulation of multiple genetic and epigenetic alterations, that confer a selective reproductive advantage to a clone, within a genetically unstable heterogeneous cell population

[2, 4, 5]. The survival and the expansion of the neoplastic cell clone are supported by the surrounding tissue that sustains and favours these conditions [6, 7]. Tumor-associated stroma actively fuels the colon cancer progression [6]. Among the tumor-associated cells are endothelial cells and pericytes that form the neo-vasculature. In turn, the newly formed angiogenic blood vessels supply tumor cells with nutrients and oxygen. In addition, mesenchymal cells are present in the stroma at earlier tumor stages, suggesting a co-evolution between stromal and cancer cells that leads to clonal expansion and metastasis [7]. Chronic inflammation, such as inflammatory bowel disease may predispose to malignancy and tumor progression. The chronic inflammatory microenvironment consists of immune, inflammatory and stromal cells, all of which produce cytokines, growth factors and adhesion molecules that may sustain tumor growth, its progression and spreading [8, 9].

The cytokines and growth factors produced by cancer cells function to create optimal growth conditions within the tumor microenvironment, while cytokines secreted by stromal cells may influence the behaviour of malignant cells. Pro-inflammatory cytokines (IL-6, IL-1) [10–12], growth factors (such as VEGF, TGF-β-1, -2, -3) and their cognate receptors [13–16] either in cancer and in stromal cells, influence not only primary gene transcription, but activate several pathways that cooperate to the aberrant clone survival, tumor expansion and metastasis [6, 10, 12]. In addition, alteration of microenvironmental factors induced by hypoxia, represents an hallmark of cancer progression [17, 18].

In this review, we discuss the role played by miRNA in colon cancer initiation and progression, especially in the context of the synergism existing among hypoxic condition, expression of IL-6 pro-inflammatory cytokine and up-regulation of VEGFA165 [19]. We report that this cooperation could act on the neoplastic cell, also by the dysregulation of miRNAs involved in colon cancer progression relevant pathways. Both the over-expression and the silencing of specific miRNAs are associated with the development and progression of colorectal cancer [20].

Recent studies have identified specific miRNAs present in colorectal cancer tissues and blood that may aid the diagnosis of cancer and help to predict disease recurrence [20–22]. Such a role in oncogenesis suggested that miRNAs could represent important targets for gene therapies. Although little is known about the exogenous factors that interfere with the regulation of miRNA expression, the microenvironment seems to play an essential role, affecting the epigenetic mechanisms that modulate miRNA gene expression, including methylation, histone deacetylation, or influencing the function of proteins involved in the maturation processing of microRNAs [23, 24]. The present review will focus on the peculiar relationship between colon cancer cells and microenvironment, that could influence miRNAs dysregulation, the apoptosis escaping and the metabolic shift, leading to invasion and metastasis.

miRNA Dysregulation and Histone Hypo-acetylation: A "Must" of the Metastatic Cell

MicroRNAs (miRNAs) are ~22-nt small noncoding RNAs that are processed from larger (80-nt) precursor hairpins by the RNase III enzyme Dicer into miRNA:miRNA duplexes. One strand of these duplexes associates with the RNA-induced silencing complex (RISC), whereas the other is generally degraded. The miRNA–RISC complex targets messenger RNAs triggering either translational repression or mRNA degradation [25]. The total number of miRNAs that are encoded in the human genome remains to be clarified. Initial estimates suggested there were up to 255 human miRNAs, but cloning and bioinformatic analyses have demonstrated that there are numerous nonconserved human miRNAs and suggest this number may be significantly larger [26]. Thousands of mammalian messenger RNAs are under selective pressure to maintain nucleotide sites matching microRNAs. Usually conserved targets are often highly expressed at developmental stages, before miRNAs expression and their levels tend to fall as the miRNA that targets them begins to accumulate. The phenomenon of the selective avoidance extended to thousand of genes enables temporal and tissue specific miRNA expression pattern, playing an important role in developmental timing [27]. It seems that the acquired differentiation and specialization is profoundly influenced by the impact of microRNA on mRNA repression and evolution. Overall, in a well differentiated tissue miRNAs are destabilizing many target messages to define tissue-specific transcript profile, but other targets could be modulated at translational level, without mRNA degradation. In that regard, changes of microenvironmental factors (as seen during cancer development) could accurately modulate protein expression patterns, acting on miRNA expression. A primary consequence in the transition from differentiated normal to de-differentiated neoplastic cell is the down modulation of miRNAs, involved in tissue specific differentiation. In humans, aberrant expression of miRNAs contributes to carcinogenesis by promoting the expression of proto-oncogenes or by inhibiting the expression of tumor suppressor genes. Such oncomirs have been demonstrated in a variety of haematological and solid tumors [28] and they contribute to cancer development and progression [20–22]. Recently, the altered miRNAs expression in colon cancer has been reported (Table 1). However, the role that miRNAs play in the development of metastasis is more poorly defined [29]. It

Table 1 miRNAs dys-regulated in colorectal cancer

	Human miRNAs	References
Down-regulated	Let-7 family	[59]
	miR-29	[66, 68]
	miR-34a	[60]
	miR-143	[61]
	miR-145	[67]
	miR-663 (targeting TGF-β1)	[64]
	miR-31	[65]
Up-regulated	miR-106b-93-25	[69]
	miR-155	[70]
	miR-21 (epigenetic switch)	[62, 63, 66]
	miR-181b-1 (epigenetic switch)	[62]
Hypermethylated	miR-124	[71, 72]
	miR-34b	[60, 71, 72]
	miR-137 (epigenetic silencing)	[24]

is worth of note that the same miRNAs could exert tumor suppressor or oncogenic effects, depending on the tumoral context and the stage of neoplastic disease. Recently, Arndt et al. [67] have shown that the re-expression of miR-143 or miR-145 leads to tumor suppressor and oncogenic phenotypes respectively, in a metastatic CRC model. In particular miR-145, reported as a 'tumor suppressor' in a non-metastatic context, showed oncogenic effects associated with the down-regulation of the G1/S cell cycle checkpoint and neuregulin pathways in the CRC metastatic setting.

The cancer microenvironment could contribute to miRNAs dysregulation affecting the epigenetic mechanisms (such as DNA methylation or histone acetylation) that regulate miRNA expression or influencing the maturation process of microRNAs [23, 24, 30].

Cancer Microenvironment and Development of Metastases: The Role of VEGF-A165a and IL-6

As previously reported [31], DNA repair failure and cell survival represent the first step in colon cancer expansion. The pro-inflammatory cytokine IL-6 seems to play a role in the inactivation of DNA repair mechanisms and Bax-dependent apoptosis. Although data on the relationship between IL-6 production and tumor progression are still conflicting, recent studies suggest a pathogenetic function of the complexes formed between IL-6 and its soluble receptor (sIL-6R), in colon carcinoma. It seems that an increased formation of IL-6-sIL-6R complexes, able to interact with gp130 on the cell membrane (trans-signaling), leads to an increased expression and nuclear translocation of STAT3, which can cause the induction of anti-apoptotic genes, such as the Bax antagonist Bcl-xL protein [10, 32,

33]. Moreover, it has been observed that in critical conditions (hypoxia, glucose deprivation, oxidative stress), the activation of STAT3, together with increased levels of HIF1α (triggered by the hypoxic condition, 1% pO2) influences the preferential expression of VEGF-A165a isoform [17, 18, 33], that could lead to the inhibition of programmed cell death inducing anti-apoptotic Bcl-2 protein. Two different splicing isoforms of VEGF165 have been identified, exerting pro- or antiangiogenic actions respectively and whose formation is strictly controlled by micro-environmental factors [34]. The production of pro- or anti angiogenic forms of VEGF depends upon splice site choice in the C-terminal. Proxymal splice site selection in exon 8 generates pro-angiogenic isoforms such as VEGF-A165a, and distal splice site selection results in the antagonistic anti-angiogenic isoforms the VEGF-A165b. Cellular choice on splice site selection, strongly influenced by microenviromental factors, depends upon the activity of RNA-binding splice factors, such as ASF/SF2. Recent studies showed a nuclear and cytoplasmic localization of ASF/SF2 splicing factor and correlated the cytoplasmic localization to the inhibition of the proangiogenic form and to a strong increase of the anti-angiogenic isoform b [34, 35].

In colon cancer, it seems reasonable that the cooperative interaction between IL-6 and increased levels of HIF1α favours the shift versus the VEGF-A165 pro-angiogenic isoforms, all these factors contributing ultimately to tumor cell proliferation, apoptotic escaping and cell migration. Along these lines, we found an increase of IL-6 protein levels in human colon cancer tissues and the increase was positively correlated with the tumor stage [36]. IL-6, released as by the tumor itself as by tumor associated macrophages (TAMs), together with IL-6-induced VEGF-A, could influence tumor cell survival interfering with the Ku-CLU-Bax physical interactions. In a colon cancer progression model, we had previously demonstrated that IL-6 increased the expression of s-Clusterin (sCLU), a multifaceted protein strongly involved in cell survival, apoptosis escaping and metastasis [36–39]. In vitro experiments have showed that the Ku-CLU-Bax interactions are modulated by IL-6, and IL-6 together with VEGF-A165 inhibit Bax-dependent cell death increasing the production of the clusterin pro-survival form (sCLU), finally shifting death into survival. In a moderately differentiated colon cancer cell line IL-6 down-modulated Bax expression at mRNA level. Concomitantly, IL-6 exposure influenced Bax at protein level acting on Bax-Ku70-sCLU physical interactions in the cytoplasm. In particular, it affected the acetylation and phosphorylation state of Ku70 [36]. The in vitro treatment with IL-6 and VEGF-A165 modulated the expression of genes involved in tumor invasion and apoptosis, as observed by microarray analysis [36]. In

particular, microarray data showed that IL-6 could influence cell proliferation, not only by inducing AP1 formation, but also through the up regulation of RASp21 protein activator 1, myc and NFκB. On the other hand, IL-6 down-modulated the expression of genes involved in the inhibition of metastasis, such as TIMP1 metallopeptidase inhibitor 1 and KISS1 [40, 41]. These still unclear molecular interactions, underline the relevant role of the microenvironmental factors in the complicated cross talk among molecules that could effectively turn the cell fate, by a fine regulation of gene expression, also acting on miRNA expression.

VEGF-A165a and IL-6: A Microenvironment Cooperation Leading to Metastasis Through MicroRNA Dysregulation

Changes of the microenvironment mediators induced by hypoxia, represent an hallmark of cancer progression. In the tumoral context, the transcriptional regulator hypoxia-inducible factor (HIF1α), cooperates with IL-6, TGF-β and VEGF-A165 in the promotion of tumoral growth [10, 15, 17–19].

The activation of hypoxia fosters neo-angiogenesis also regulating the expression of miR-210, and its target Ephrin-A3, involved in tube formation and survival of endothelial cells [42]. A group of candidate miRNAs regulated by hypoxia have been recently identified: miR-16, 20let-7b, miR-17-5p, miR-27, miR-106, miR-107 miR-193, miR-210, miR-320 and miR-361, showing VEGF as potential target [43, 73]. Moreover, using a bioinformatic prediction program, several key genes of proliferation, metabolism, migration and apoptotic response (miR-23, miR-26, miR-27, miR-30, miR-181) were found to be potentially targets of hypoxia-regulated miRNAs [57, 58]. In the colon cancer context, the synergism among hypoxic condition (HIF-1α activation) and IL-6 pro-inflammatory cytokine induces the up-regulation of VEGF-A165, contributing to neoangiogenesis and metastasis formation.

In colon cancer cells (Caco-2), we observed that the synergism of IL-6 and VEGF-A165 influenced the expression of tumor suppressor miRNAs, involved in epigenetic control and epithelial to mesenchymal transition (EMT), which strongly correlated to the *malignization* of many types of cancers [6]. In particular, the in vitro co-treatment with IL-6 and VEGF-A165 significantly down modulated miR-619, which targets VEGF gene expression determining a positive feed-back loop, finally contributing to cancer growth and spreading [44]. In addition, a significant decrease of miR-200 (12-folds decrease) was found, as compared to untreated colon cancer cells. The target prediction program indicates that highly conserved binding sites for the miRNA200 family are present in the ZEB1 and SIP1 mRNAs, which are repressors of ecadherin, implicated in EMT transition and tumor metastasis. The same down-regulation of miR-200

was obtained in response to TGF-β treatment and this effect was sufficient to induce EMT, in a process requiring up-regulation of ZEB1 and/or SIP1 [45]. In addition, Adam et al. [46] demonstrated that miR-200 controls the sensitivity to the EGFR therapy, which represents an actual issue in the treatment of human highly aggressive and metastatic colorectal cancer. In fact the expression of the miR-200 is sufficient to restore the EGFR dependency in bladder cancer cells, targeting ERRFI-1 which is a novel regulator of EGFR-independent growth.

In the last years, great attention has been given to the development of target anti-cancer therapies, based on the knowledge of transcriptional mechanisms epigenetically regulated in tumoral tissues. Moreover, it has been recently delineated a reciprocal interconnection between microRNAs and epigenetics [30]. The epigenetic modifications, such as DNA methylation or histone acetylation, are able to affect miRNAs expression causing oncomirs dysregulation, but the same microRNA (epi-miRNAs) may directly control the epigenetic machinery targeting its enzymatic components [30].

Evidence of the epigenetic influence of the microenvironment on tumoral cells has been observed by co-treating colon cancer cells with IL-6 and VEGF-A165. A strong down-modulation of the growth suppressing miR-449, which targets histone deacetylase 1 molecule (HDAC-1) [47] has been found in our system. This enzyme plays a crucial role in the epigenetic control and display high significance in oncology [48, 49]. Aberrant HDAC level and/or sustained activity has been found in various types of cancer and metastasis [50]. It has known that recruitment and activation of HDACs results in tightening of chromatin structure, thus the transcriptional machinery is prevented from accessing the DNA. Hence, cytokines and growth factors in the microenvironment could modulate gene transcription at epigenetic level, affecting epi-miRNA (as miR-449 [47]), or influencing the activity of protein involved in the regulation of HDAC activity (Fig. 1).

miRNAs Expression and Altered Metabolism in Cancer

The altered metabolism of tumor cells may be a potential means by which these cells evade programmed cell death, favoring survival and tumoral growth. In particular, the metabolism of fatty acids is markedly altered in the tumoral context. It has been demonstrated that the extracellular acidosis in the microenvironment of solid tumors can work in an epigenetic fashion by up-regulating the transcriptional expression of fatty acids synthase (FASN) gene, proposed as the "metabolic oncogene" of cancer cells [51].

Recent reports demonstrated the existence of miRNAs able to recognize and modulate the transcriptional levels of metabolic factors, relevant both in non neoplastic and in
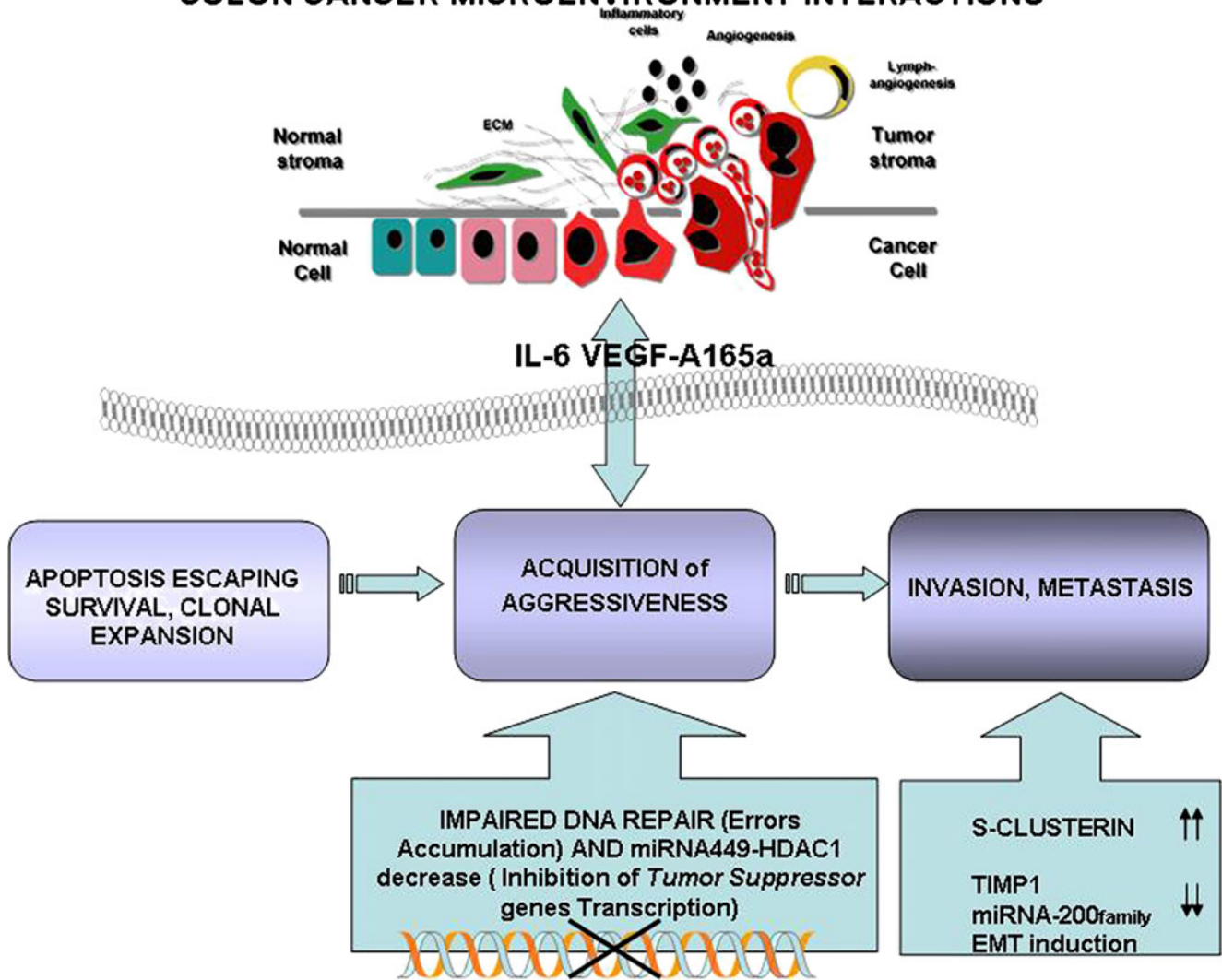
COLON CANCER MICROENVIRONMENT INTERACTIONS

Fig. 1 Cancer- stroma interactions in colon cancer progression and spreading. In colon cancer the synergistic action of IL-6, and VEGF-A165a promotes the acquisition of aggressiveness regulating the DNA repair capacity of the cell, whose impairment is the first step of colon cancer development. The cooperative action of these soluble media-tors could modulate cancer-related gene transcription (sClusterin, TIMP1), and affect the expression of small non coding mRNA (microRNAs), able to modulate the epigenetic machinery (epi-miRNAs; miR-449) and to induce the epithelial to mesenchimal transition (EMT induction; miR-200 family)

cancer cell. In particular, transfection experiments with sense and antisense sequences displayed that the increase of lipogenesis in HepG2 cells (hepatocarcinoma cell line) is directly controlled by miR-122 [52]. This miRNA sequence is up-regulated by miR-370, the last therefore indirectly involved in the upregulation of lipogenic genes. Interest-ingly the same miR-370 targets the 3′UTR of CPT1, down-regulating the expression of CPT1A gene and the rate of β-oxidation [52].

Carnitine palmitoyl transferase 1 (CPT1) is another limiting factor in the fatty acids metabolism and it is contra-regulated by FASN substrate malonyl-CoA [53]. CPT1 resides physiologically at the mitochondrial mem-brane transporting long-chain fatty acids for β-oxidation.

Recently, a CPT1A transcript splice variant, termed variant 2 (NM_001031847), was identified in colon cancer and breast cancer cells and it was undetectable in the corresponding non neoplastic cell line [53]. This variant codifies for a protein which differs in only 11 aminoacids from CPT1A variant 1, at the C-terminus. The preferential cellular localization of the two CPT1A variants was identified, being the product of transcript variant 2 mainly localized in the nucleus of tumoral cells.

The two transcript variants of CPT1A show great similarity differing only at the 3′-UTR level, where the recognition with miR-370 is located [52]. A different effectiveness of miR-370 in the target recognition could be responsible for the different levels of CPT1A transcripts

found in cancer cell, as compared with non neoplastic cell. In fact, miR-370 could preferentially target CPT1 isoform 1 and indirectly favour CPT1 isoform 2, localized in the nucleus of cancer cells.

Experiments performed on human colorectal and breast tumoral tissues and cancer cell lines (Caco-2, HepG2 and MCF-7 cells) showed that the nuclear localization of CPT1A correlated to an increased HDAC activity and a resulting decrease of histone acetylation [53]. Moreover, HDAC-1 protein co-precipitated with CPT1A.

We have evidence of a different binding affinity of the two CPT1A variants for HDAC1. These data are based on the dissimilar CPT1 residues at the interface with HDAC1, for the two complexes. In the case of isoform 2 the interaction with HDAC1 involved a region close to the C-terminus, where the differences with CPT1 isoform 1 are located. Considering the great similarity of the other regions of CPT1 isoforms, this evidence suggested a higher stability of the complex CPT1 isoform2/HDAC1 (unpublished data). HDAC complexes are recruited to specific genomic sites by regulatory proteins. Additional data are needed to confirm if CPT1 (and in particular the nuclear isoform) could function as a novel partner of HDAC-1, stabilizing HDAC complexes at acetylated-histone tails of specific promoters, to silence tumor suppressor genes involved in the control of cancer cell growth and migration.

Altered Metabolism and Cancer Microenvironment

Experimental evidence suggest that, in tumoral context, the modulation of the metabolic factors could be driven by miRNA target recognition [52], but also through the activation of membrane receptors signalling, such as EGF/EGFR family, induced by microenvironmental stimuli [51, 54].

The human epidermal growth factor receptor type 1 (EGFR/HER-1) gene encodes a membrane receptor protein in the epidermal growth factor receptor family (ERBBs) and evidence suggests that EGFR expression and its activation status are associated with hyper-proliferation of colon cancer cells. Increased expression of EGFR is independently associated with poor disease-free survival after adjustment for stage, histological grade, age, and DNA mismatch repair status [55]. Epidermal growth factor and the EGF receptors (known as ERBB1) and ERBB2 have been shown to stimulate fatty acids synthase (FASN), the major enzyme required for the synthesis of fatty acids, although the ultimate mechanisms responsible for tumour-associated FASN overexpression are not completely understood. The effects of growth factors and growth factors receptors on FASN are complex and involve activation and/or cross-talk between multiple signal-transduction pathways

[56] The EGFR activation found in colorectal cancer [55] could induce an up-regulation of FASN, determining the down-regulation of CPT1A activity in mitochondria [53].

Overall, in the neoplastic cells the gradient of microenvironmental factors (including EGF/EGFR activation) induces the overexpression of FASN and the resulting inhibition of fatty acids β-oxidation. Thus, a differential expression of CPT1A isoforms, driven by miRNA target recognition [52], could favour the CPT1 nuclear variant that evidence suggest to be implicated in the epigenetic regulation [53].

In conclusion, the metabolic factors altered in cancer could directly regulate the expression of genes implicated in tumor development and metastases, or modulate at epigenetic level the expression of small non coding RNAs. Besides, microRNAs have been identified (and many others remain to be uncovered) that target metabolic factors modulating their expression. Once more this regulatory loop seems to be driven by microenvironmental factors.

Conclusion and Perspectives

Invasion and metastasis are the deadly face of malignant tumors. Considering the high rate of incidence and mortality of colorectal cancer, it is critical to determine the mechanisms of its dissemination. The outcome of this non random process depends, in part, on the interaction of unique tumor cells with a compatible organ microenvironment. On the other hand, overexpression and silencing of specific miRNAs are associated with the development and progression of colorectal cancer. It is worth of note that miRNA dysregulation could play opposite role in the early stages of colon cancer, as compared to a metastatic context. As reported above, the pathway analyses may explain the observed oncogenic effects of miR-145 in metastatic CRC, compared to its reported tumor suppressor effects in the non-metastatic context [67].

The present review attempts to shed light on the fine relationship between altered gene expression profiles implicated in colon cancer progression and regulatory miRNAs, induced by pro-inflammatory partners of tumor microenvironment. Furthermore, in the neoplastic cells the gradient of microenvironmental factors induces the alteration of metabolic enzymes, such as FASN and CPT1A, which act on the epigenome modulating miRNA expression. The other side of the coin is represented by the identification of miRNAs which target the same metabolic factors, modulating their expression levels. Nowadays, the identification of specific miRNAs directly involved in cancer spread and metastasis may signify novel diagnostic tool in the characterization of gene targets.

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