Malay Chatterjee *Editor*

# Molecular Targets and Strategies in Cancer Prevention

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# Contents

# Strategies to Target Pancreatic Cancer

**Geou-Yarh Liou and Peter Storz**

## 1 Introduction

Pancreatic cancer is an extremely lethal type of cancer with a 5 year survival rate of approximately 5–6 %, and this has not been substantially improved since 1970. Based on the pancreatic cell type they originate from, pancreatic cancers can be divided into endocrine and exocrine cancers. Incidents of pancreatic endocrine cancers are relatively rare as compared to pancreatic exocrine cancers. Endocrine cancers comprise pancreatic neuroendocrine cancer or islet cell carcinomas and affect hormone-producing cells. In comparison to exocrine cancers, endocrine cancers grow relatively slow, however, they still result in high mortality rates. Approximately 95 % of pancreatic cancers are exocrine cancers [1, 2]. They affect the pancreatic functions to secret and deliver digestive enzymes to the gastrointestinal tracts. Pancreatic exocrine cancers usually are pancreatic ductal adenocarcinomas (PDA). This chapter mainly will focus on these cancers.

More than 95 % of PDA harbor oncogenic Kras mutations, suggesting that pancreatic cancer originates as a genetic disorder. Studies using genetic animal models have shown that while activating mutations in Kras can initiate formation of pancreatic pre-neoplastic lesions, in order to further progress to carcinoma *in situ* or metastatic PDA, tumor cells need to acquire additional mutations. These include inactivation of tumor suppressor genes such as *TP53*, and acquisition of increased telomerase activity [1–3]. In addition, inflammation of the pancreas in response to pancreatitis was demonstrated to increase the risk of developing PDA. Other "lifestyle" risk factors for pancreatic cancer that may induce inflammation include smoking, obesity and diabetes [4–6].

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Pancreatic cancer usually is detected at a late stage, when it already is metastatic. Therefore, a critical issue for increasing patient survival is to develop new methods for early detection of tumor development or cancer cell dissemination. Identification of early pancreatic cancer markers in patients (i.e. by gene panel analysis along with regular blood screening tests to early detect circulating pancreatic cancer cells) would allow an earlier intervention. A problem for treatment of patients diagnosed with PDA is that current chemotherapeutics, although they may work well *in vitro* in cell culture, because of the desmoplastic reaction in patients, fail to be effective *in vivo*. Therefore, a key issue for efficient treatment is to develop strategies that overcome desmoplasia and drug resistance and make tumor cells accessible to conventional chemotherapy.

## 2 Genetics of Pancreatic Cancer

Genetic predispositions to develop pancreatic cancer have been identified in patients with familial pancreatic cancer. Moreover, inherited gene mutations, metabolic disease or lifestyle leading to chronic pancreatitis are risk factors. However, such genetic or lifestyle predisposition alone does not necessarily lead to pancreatic cancer, since additional gene mutations need to be acquired (Fig. 1).

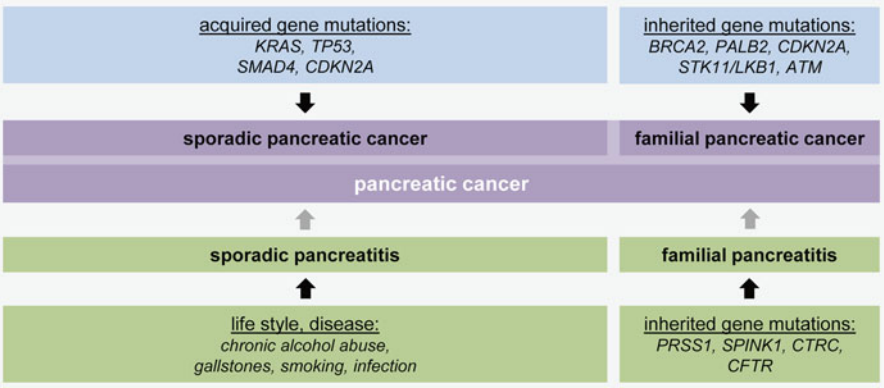


**Fig. 1** Gene mutations specific to pancreatitis and pancreatic cancer. Gene mutations that can lead to development of pancreatic cancer are either acquired or inherited. Most prominent examples for acquired gene mutations leading to sporadic pancreatic cancer are *KRAS* activating mutations or deletion of tumor suppressor genes such as *TP53*. Most prominent gene mutations leading to familial pancreatic cancer are deletion or inactivation of tumor suppressor genes such as *BRCA2* and *STK11*/*LKB1* for inherited gene mutations. Chronic pancreatitis is a risk factor for pancreatic cancer and can be caused by inherited gene mutations (familial pancreatitis) or by other insults such as chronic alcohol abuse, smoking and gallstones. *Black arrow*: leads to the indicated disease; *gray arrow*: risk factor for the onset of disease

## 2.1 Hereditary/Familial Pancreatic Cancer

Familial pancreatic cancer accounts for 5–10 % of all occurrences. *BRCA2* was the first gene that was linked to familial pancreatic cancer. *BRCA2* is a tumor suppressor gene, responsible for DNA repair and its mutations are frequently found in breast cancers. Similarly, in familial pancreatic cancer, *BRCA2* deletion mutations, point mutations and frameshift mutations have been reported [7, 8]. In addition, in some hereditary pancreatic cancers truncating mutations of *PALB2* were reported [9]. Its gene product PALB2 is the nuclear binding partner for BRCA2; and PALB2/BRCA2 complexes are required for efficient DNA repair and cell cycle control [9, 10]. Interestingly, *PALB2* truncating mutations are also associated with familial breast cancer patients, indicating the importance of a functional DNA repair system for preventing the occurrence of cancer in general [11–13].

Another regulator of cell cycle progression that has been linked to familial pancreatic cancer is *CDKN2A* [14]. Due to transcription variants, *CDKN2A* encodes both the cyclin-dependent kinase inhibitor $p16^{INK4a}$ and the p53 activator $p14^{ARF}$, which both regulate cell cycle progression. In addition to developing pancreatic cancer, patients with inherited mutations in $p16^{INK4a}$ also suffer from melanoma [15–17]. Inactivating mutations of *CDKN2A* are also commonly found in sporadic pancreatic cancer patients accounting for 95 % cases, indicating a key role of *CDKN2A* in pancreatic tumorigenesis.

Germline mutations in *STK11*/*LKB1* cause Peutz-Jegher syndrome (PJS), an autosomal-dominant disorder characterized by the development of benign hamartomatous polyps in the gastrointestinal tract and hyperpigmented macules on the lips and oral mucosa. The *STK11*/*LKB1* gene product LKB1 mediates apoptosis by its interaction with p53 and this requires its translocation to the mitochondria [18]. It has also been demonstrated that LKB1 regulates cell cycle arrest by interacting with Brg1 in SWI/SNF chromatin-remodeling complexes or by inducing the cyclin-dependent kinase inhibitor $p21^{WAF1}$ [19, 20]. Inactivating mutations in *LKB1* found in PJS patients has been shown to incline the likelihood to develop early-onset pancreatic and biliary adenocarcinomas [21], possibly due to an imbalance between proliferation and apoptosis in the absence of functional LKB1 in the gastrointestinal cells.

Multiple gene mutations in the ataxia telangiectasia (*ATM*) gene have been identified to correlate with familial pancreatic cancer [22–24]. All of these deleterious mutations result in loss of function of ATM, a protein kinase involved in DNA double-strand break repair. Similar as seen for mutations of *CDKN2A* or *STK11*/*LKB1*, patients with ATM mutations not only have an increased risk of pancreatic cancer but also are predisposed to other types of cancer. In case of ATM these include lymphoma, leukemia and breast cancer [25–28].

## 2.2 Signature Mutations Found in Sporadic Pancreatic Cancer

Global genome analysis of human pancreatic cancers and confirmation with transgene and knockout mouse models revealed a number of sequential signature events leading to development and progression of PDA. These include activating mutations in *KRAS* and inactivation of the tumor suppressor genes *CDKN2A*, *TP53* and *SMAD4* [10, 29, 30]. The mutations found in these genes are associated with several core signaling pathways (e.g. Hedgehog signaling, integrin signaling, Wnt/Notch signaling, Kras signaling, TGFβ signaling and small GTPase dependent signaling) that have been involved in cancer development and progression by regulating apoptosis, DNA damage control, cell cycle checkpoints and proliferation as well as invasiveness [10].

Eventually, pancreatic cancer cells acquire chromosomal abnormalities that are caused by telomere dysfunction and centrosome aberrations. Parallel paired-end sequencing on human pancreatic adenocarcinoma samples also indicated that genomic DNA rearrangements occur frequently and early in development of pancreatic cancer [31]. Among these rearrangements, deletions and fold-back inversions dominate other types of aberrations such as tandem duplications and amplicon-related rearrangements, suggesting that the presence of a dysregulation of the G1-to-S transition without halting a G2-M checkpoint in pancreatic cancer cells leads to genomic instability.

The most prevalent gene mutations which result in development of sporadic pancreatic cancer are activating mutations in *KRAS* (occurring in approximately 95 % of human PDA). Therefore, development of small compounds to directly target oncogenic Kras and/or its downstream signaling pathways remains of tremendous interest in the pancreatic cancer research field. Among the Kras activating mutations, a replacement of Gly12 with Asp, is the most common found in PDA. Such a $Kras^{G12D}$ mutation locks Kras in a constitutively-active GTP-bound state, allowing persistently transmitting signals to downstream targets that potentiate proliferation such as ERK/MAPK and PI3K [32–34].

Using genetically-engineered mouse models in which $Kras^{G12D}$ is specifically expressed in cells of the pancreas, it has been demonstrated that occurrence of oncogenic Kras is essential for the formation of pancreatic intraepithelial neoplastic lesions (PanINs) [35–37]. Evidence from mouse models combined with lineage tracing and genetic labeling suggested that PanINs arise from acinar cells expressing oncogenic Kras or aberrant epidermal growth factor receptor 2 (EGFR2) signaling [38–42]. These acinar cells undergo a transdifferentiation to a duct-like progenitor phenotype after activation of Sox9 [39, 43]. Resulting cells are believed to progress to low grade PanIN lesions. Although the PanIN

lesions that occur after acquisition of mutant Kras are of low grade and usually do not progress to carcinoma *in situ*, their detection and targeting could be a preventive care for patients with a hereditary high risk for developing PDA.

Only when additional gene mutations are acquired or in presence of pancreatic inflammation such low grade PanINs progress to carcinoma *in situ* (PanIN3) or PDA. Compelling evidence from transgenic mouse models revealed that inactivation of tumor suppressor genes such as *CDKN2A*, *TP53* and *SMAD4* over time drives such progression [44–49]. The presence of chronic pancreatic inflammation (pancreatitis) also has been shown to be essential for progression of Kras-caused lesions to PDA [50, 51].

## 2.3 Inherited Mutations That Increase the Risk of Developing Pancreatic Cancer

Chronic pancreatitis is a risk factor for developing pancreatic cancer. Pancreatitis is caused by release of digestive enzymes from pancreatic acinar cells leading to autodigestion [52, 53]. Chronic pancreatitis can result from gallstones, heavy alcohol use, metabolic disorders and bacterial or viral infections, but also can have a genetic background. Germline mutations in several protease genes that encode for enzymes secreted by acini to help digest food have been associated with hereditary pancreatitis. The population with such hereditary pancreatitis has an extremely high risk (35-fold or greater by age of 70–75) for developing pancreatic cancer.

The most prominent gene mutated in hereditary pancreatitis is *PRSS1*. Its gene product is cationic trypsinogen, which is enriched in pancreatic acinar cells. More than 40 different *PRSS1* mutations have been unveiled in hereditary pancreatitis patients [54–56], and the most common ones are R122H and N29I [57, 58]. All of these *PRSS1* mutations either lead to a persistent enzymatic activity of trypsinogen regardless of its localization, or prevent its degradation. Results from a *PRSS1* transgenic mouse model demonstrated that overexpression of a R122H mutant in mouse pancreatic acini results in early onset cell injury and immune cell infiltration. Moreover, with progressing age, these mice developed pancreatic fibrosis and dedifferentiation of pancreatic acini [59].

Other inherent gene mutations associated with familial or idiopathic pancreatitis have been described in *SPINK1* [60, 61], *CTRC* [62], and *CFTR* [63, 64]. *SPINK1* encodes a pancreatic secretory trypsin inhibitor and its mutations can result in a reduced inhibitory capacity or a decreased secretion of SPINK1 to inefficiently suppress trypsin activity [65]. *CTRC* encodes the digestive proenzyme chymotrypsinogen C to regulate trypsinogen activation and degradation. The mutations of *CTRC* associated with pancreatitis result in a diminished secretion, or loss of catalytic activity of chymotrypsinogen C [62, 66, 67]. The cystic fibrosis transmembrane receptor (CFTR) is an ion channel participating

in the process of transporting chloride and thiocyanate. *CFTR* mutations not only lead to cystic fibrosis but also can contribute to chronic pancreatitis. It has been demonstrated that these mutations reduce chloride secretion and abolish bicarbonate secretion across ductal epithelial cells to cause pancreatic ductal obstruction, eventually leading to pancreatitis [68–70].

## 3 Biomarkers for Detecting Pancreatic Cancer at an Early Stage

Pancreatic cancer usually is detected in patients by computed tomography (CT scan), magnetic resource imaging (MRI) and positron emission tomography (PET scan) followed by further confirmation through patient tissue biopsy. Only a very small number of pancreatic cancer patients have localized tumors that can be removed by surgical treatment such as a Whipple operation. Most patients are diagnosed with stage IV pancreatic cancer, suggesting that tumor cells already have spread into lymph nodes or the blood stream. Disseminated tumor cells not only account for the high mortality in pancreatic cancer patients, but also can cause later tumor recurrence even if patients survive the first 5 years after diagnosis and treatment. Recent work has shown that analyses of circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA), or the microRNA (miRNA) expression profile

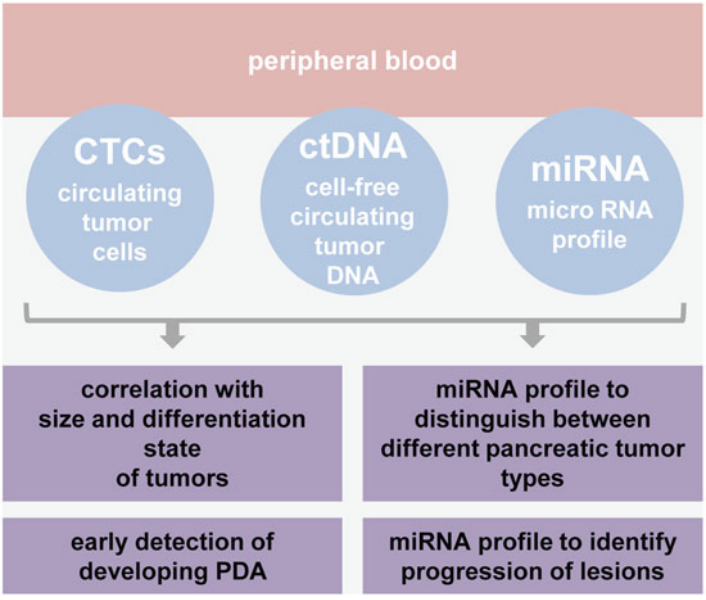


**Fig. 2** Possibilities for early detection and stage of tumors. Ideally, body fluids such as peripheral blood could be a source for detecting early markers for tumor formation or presence and stage of tumors. Possibilities for such analyses could be to capture circulating tumor cells (CTCs), to analyze circulating tumor DNA (ctDNA) or to determine tumor specific microRNA profiles

from patient blood samples may allow early detection or may provide feedback on treatment response (Fig. 2).

### *3.1 Circulating Tumor Cells as Markers for Early Detection and Progression*

A possible way to decrease pancreatic cancer-caused death is the early detection of circulating tumor cells (CTCs). Both, biomarkers specific for detection of CTCs, as well as highly sensitive detection techniques are needed. Since circulating tumor cells in peripheral blood are present at a very low frequency, several approaches including density gradient centrifugation, membrane filtration and immune-magnetic beads are utilized to enrich circulating tumor cells prior to detection [71–73]. Markers to capture pancreatic CTCs usually are epithelial surface markers such as EGFR, cytokeratin-19 (CK-19), or carbohydrate antigen 19-9 (CA 19-9).

In pancreatic cancer patients, oncogenic Kras mutations as well as epigenetic modifications can be identified by DNA analyses of CTC from peripheral blood, providing useful information for assessing tumor burden and efficacy of treatment in patients [74–76]. Several studies also have demonstrated the possibility of detecting circulating pancreatic cancer cells from patients' peripheral blood by combining enrichment steps with highly-sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assays [77–80]. Markers used for RT-PCR detection of CTC include EGFR, CK-19, CA 19-9 and carcinoembryonic antigen (CEA). In particular, due to its high sensitivity CK-19 is a commonly used maker for RT-PCR-mediated detection of circulating tumor cells from samples of blood, bone marrow and biological fluids. It has been demonstrated that CK-19 can be detected from disseminating pancreatic cancer cells in blood, bone marrow and peritoneal lavage [78]. Moreover, the mRNA level of CK-19 from peritoneal lavage samples has been shown to correlate with size and differentiation state of pancreatic tumors. However, the procedure to obtain peritoneal lavage is relatively invasive which makes it impossible to develop this method as a routine screening test for high risk populations.

Tumor cell metastasis requires that cells undergo epithelial-mesenchymal transition (EMT) to become invasive and break through the basement membrane and eventually enter the bloodstream (intravasation). As shown by a recent study using lineage tracing to visualize cells in preneoplastic lesions, EMT and dissemination into the bloodstream can occur at a PanIN stage, indicating that these cells metastasize to other organs even before PDA develops [81]. Moreover, upon enrichment these circulating tumor cells can be detected in the majority of pancreatic cancer patients, but also in 33 % of patients with pancreas cystic lesions but without clinic diagnosis of cancer [79]. This suggests that detection of these early disseminating cells could serve as earliest markers for development of PDA.

### 3.2 Cell-Free Circulating Tumor DNA as Marker for Early Detection

In addition to circulating tumor cells, cell-free circulating tumor DNA (ctDNA) is another possibility for early detection [82, 83]. ctDNA consists of small fragments of nucleic acids that are not associated with cells or cell fragments. They possibly are released from circulating tumor cells or necrotic cancer cells. Another explanation for increased occurrence of ctDNA is that tumors may actively release newly-synthesized DNA into the blood stream to interfere with the host's immune system. Detection of ctDNA in the peripheral blood samples has been evaluated for its clinical potential as a routine test for cancer patients [84–86]. For breast and lung cancer, numerous studies have demonstrated that both ctDNA and circulating tumor cells are present in the peripheral blood in patients with advanced neoplasia [87–89].

### 3.3 Profiling of Micro RNAs as a Method for Early Detection and Response to Treatment

Another method that holds great promise is the detection of microRNA (miRNA), small non-coding RNA, which plays a critical role in RNA silencing and post-transcriptional regulation of gene expression. Dying tumor cells release miRNAs which stably circulate in the peripheral blood. A number of studies have demonstrated that these circulating miRNAs in the blood of cancer patients may serve as biomarkers for early detection of tumor, treatment and prognosis (reviewed in [90]). For example, increased expression of miR-155 and miR-21 was detected in precancerous PanIN lesions and PanIN3 [91], revealing a clinical potential of both as biomarkers for screening of high risk populations prior to cancer formation. Moreover, a differential expression of miRNAs in other types of pancreatic lesions such as intraductal papillary mucinous neoplasms was revealed [91, 92]. Comprehensive profiling of miRNA expression in pancreatic cancer patient samples led to identification of miRNAs specifically-expressed in PDA, pancreatic endocrine cancer and acinar tumors [93–96].

Several miRNAs such as miR-21, miR-10b, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c and miR-205 have been reported to modulate cancer invasion and metastasis [97–100] and it may be possible to correlate their occurrence with early metastasis. In addition, since miR-155, miR-200 and miR-34 in pancreatic cancer have been shown to contribute to chemotherapeutic drug resistance by regulating cancer stem cells and/or EMT [101], detection of these miRNAs in blood samples of cancer patients who are under radiation therapy and chemotherapy could also allow monitoring treatment efficacy and predicting outcome. In summary, a comprehensive profiling of miRNAs could be a precise method for early detection of different types of pancreatic cancers, metastasis and response to treatment.

## 4 Drug Resistance and Desmoplasia in Pancreatic Cancer

When diagnosed, the majority of pancreatic cancer patients show locally-advanced, unresectable or metastatic disease. Current treatment strategies for these patients using chemotherapy and radiation therapy or combination therapy of gemcitabine with cytotoxic agents such as 5-FU, cisplatin, oxaliplatin, and capecitabine [102–104], or targeted agents such as erlotinib, cetuximab and bevacizumab [105–107] have been shown to be largely unsuccessful [108]. A cause of such failure may be the insensitivity of pancreatic tumors to therapeutic drugs due to upregulation of signaling pathways that mediate multidrug resistance, as well as the existence of highly-resistant cancer stem cells [109–114]. Another problem is the failure to deliver chemotherapeutic drugs to the cancer site, which is due to a tumor microenvironment (TME) with high stromal desmoplasia and a reduction in vessel density [115, 116]. Strategies for better drug delivery may include the depletion of the fibrotic tissue to facilitate better drug delivery [117], or the use of nanoparticle-bound or other delivery systems [118].

### *4.1 Drug Resistance Due to Regulation of ABC and HENT1 Transporters*

Drug resistance can be divided into two categories, an intrinsic (innate) resistance and an acquired resistance [119]. The intrinsic drug resistance originates from genetic alterations; and patients with this type of resistance show little to no response to therapeutic treatment. In contrast, acquired drug resistance develops over time after exposure to anticancer drugs and eventually can cause cancer relapse. Accumulating evidence indicated that upregulation of ATP-binding cassette (ABC) transporter genes contribute to drug resistance by extruding anticancer drugs from cancer cells [120–122]. ABC transporters are transmembrane proteins to transport substances across the cell membrane by utilizing ATP as energy source. The human genome contains 49 ABC genes which belong to seven subfamilies from A to G according to their domain arrangement and amino acid homology [123, 124]. Among these subfamilies, increased expression of ABCB, ABCC and ABCG can render cancer cells more resistant to chemotherapeutic drugs. For human pancreatic cancer tissues and pancreatic cancer cell lines, it was demonstrated that upregulation of ABCB1, ABCC1 and ABCG2 mediates resistance to 5-FU and gemcitabine [120, 125–127].

Another transporter family to regulate the cellular entry of anticancer drugs across the cell membrane is the human equilibrative nucleoside transporter 1 (hENT1). The sensitivity of human pancreatic cancer cell lines to 5-FU and gemcitabine is dependent on the level of hENT1 expression [128]. It also was demonstrated in a clinical trial study that pancreatic cancer patients with high levels of hENT show better response to gemcitabine than patients with low expressions [129].

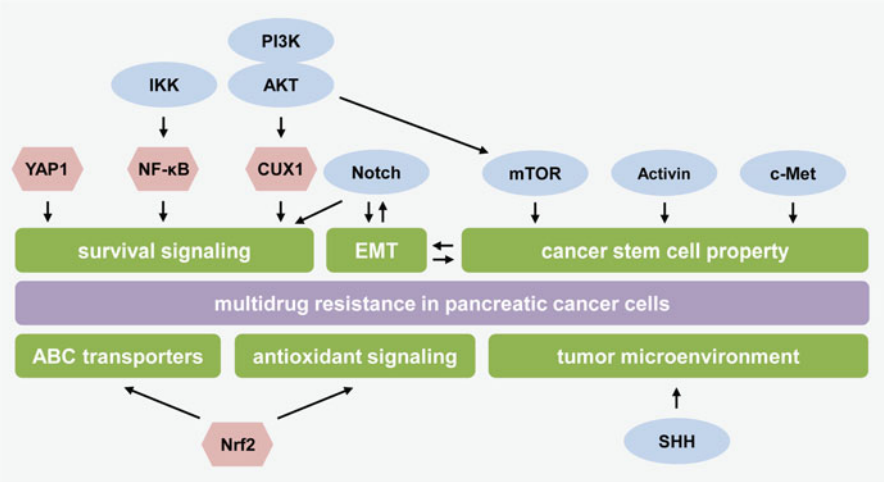


**Fig. 3** Key signaling pathways and molecules involved in multidrug resistance of pancreatic cancer cells. Several signaling molecules have been shown to increase multidrug resistance in pancreatic cancer. For example, resistance to drug-induced cell death can be achieved by activation of transcription factors such as Yap1, NF-κB, CUX1 or Notch. Notch also regulates EMT to promote stem cell formation. Similarly, activation of mTOR, activin and c-Met have been shown to increase stem cell features. Nrf2 is a transcription factor that has been shown to contribute to multidrug resistance by upregulating ABC transporters and antioxidant genes. Eventually, altered vasculature and increased fibrosis have been show to contribute to drug resistance. Targeting sonic hedgehog (SHH) can decrease such stromal density and increase accessibility of gemcitabine

## *4.2 Drug Resistance Due to Insensitivity to Cell Death*

In addition to increased expression of transport systems to exclude chemotherapeutic drugs, cancer cells often also upregulate cellular signaling pathways that counteract their mechanisms of action and protect or in-sensitize from drug-induced cell death (Fig. 3). Key signaling pathways driving these processes are regulated by phosphatidylinositol-3-kinase (PI3K)/Akt signaling, nuclear factor k-light chain-enhancer of activated B cells (NF-κB), Notch, and nuclear factor (erythroid-derived 2)-like 2 (Nrf2).

The PI3K/Akt signaling pathway regulates multiple cellular processes including apoptosis, proliferation, cell migration, glucose metabolism and angiogenesis. In pancreatic cancer Akt signaling can mediate the resistance to apoptosis by activating the transcription factor CUX1, leading to increased Bcl2 expression. Moreover, a knockdown of CUX1 attenuated pancreatic tumor growth and enhanced apoptosis in orthotopic human pancreatic cancer xenografts [130]. The inhibition of this pathway using PI3K inhibitors promoted gemcitabine-induced apoptosis in both human pancreatic cancer cells as well as in pancreatic cancer xenograft mice [130–134].

NF-κB is a transcription factor that participates in a multitude of biological processes, including cell survival, cell migration, tumorigenesis and immune response [135, 136]. In human PDA cell lines, increased activity of NF-κB was detected in apoptosis-resistant cells, and inhibition of upstream signaling re-sensitized cells to apoptotic stimuli [137]. The canonical activation for NF-κB is initiated by IκB kinase β (IKKβ)-mediated phosphorylation and proteasomal degradation of inhibitor of k-light chain-enhancer of activated B cells (IκB), a protein that sequesters NF-κB in the cytosolic compartment, resulting in nuclear RelA/p50 NF-κB complexes. The alternative activation for NF-κB is initiated by NF-κB-inducing kinase (NIK) and IKKα, resulting in processing of p100 to p52 and formation of nuclear RelB/p52 NF-κB complexes. In pancreatic cancer cell lines and patient samples constitutive basal NF-κB activity was linked to TRAF2 degradation which in turn leads to stability and activity of NIK. Such activation of alternative NF-κB signaling increased cell proliferation and colony formation of pancreatic cancer cell lines [138]. In contrast, activation of canonical NF-κB signaling was associated with gemcitabine resistance [112], and its inhibition by MG132, sulfasalazine or by overexpression of a superdominant IκB all sensitized pancreatic cancer cells to gemcitabine-induced cell death [109]. In an orthotopic mouse model, pancreatic tumors were significantly smaller when oxaliplatin treatment was combined with 3,3-diindolytmethane (DIM). This was linked to a decrease of oxaliplatin-induced NF-κB activity in tumors mediated by DIM. This suggests that blockade of NF-κB activity that is induced by chemotherapeutic drugs is a possibility to re-sensitize pancreatic tumors to conventional therapies [139, 140]. A novel approach for therapy also could be the use of zoledronic aid, which can reverse EMT and self-renewal of cancer cells through inhibition of NF-κB [141]. In human pancreatic cancer cells zoledronic acid has been shown to induce anti-proliferative and apoptotic effects, which are typically associated with blockage of NF-κB activation [142].

Notch is a transmembrane receptor that coordinates apoptosis, proliferation and differentiation during embryogenesis and tissue development. Dysregulation of Notch signaling not only leads to developmental defects but can also result in tumorigenesis. Gemcitabine-resistant pancreatic cancer cells express high levels of Notch and its ligands, Jagged and DIL [114, 143–145]. Upon binding of its ligands, ADAM proteases and γ secretases mediate the activation of Notch by cleavage, leading to the formation of NICD (Notch intracellular domain) which functions as a transcription factor that regulates genes involved in cell cycle progression and apoptosis. Consequently, the blockade of Notch signal by γ secretase inhibitors increased pancreatic cell death in response to gemcitabine treatment [145].

Nrf2 is a key transcriptional regulator of a multitude of genes that facilitate defense against oxidative stress and drug metabolism. Its expression has been shown to be upregulated in pancreatic cancer cell lines, Kras-caused preneoplastic lesions and ductal adenocarcinomas [146, 147]. Functions for Nrf2

include support of proliferation and chemoresistance. The latter is mediated by a greater intrinsic capacity of these cells to respond to toxic oxidative stress levels induced by chemotherapeutic intervention. In addition Nrf2 can regulate drug resistance in pancreatic cancer cells through upregulation of ABC transporter genes [148]. In summary, pharmacological manipulation of either levels or activity of Nrf2 may reduce pancreatic tumor growth, and increase sensitivity to therapeutics.

### *4.3 Drug Resistance and Recurrence Due to Acquisition of a Stem Cell Phenotype*

Treatment of human pancreatic cancer xenografts with ionizing radiation and gemcitabine resulted in an enrichment of cancer stem cells (CSCs) in the tumors [149]. CSCs are capable of self-renewal, production of differentiated cells and expansion of a whole population of malignant tumor cells. The presence of cancer stem cells is associated with high chemotherapy resistance and a high recurrence rate [110]. Characteristic surface markers for pancreatic cancer stem cells include CD44, CD24, epithelial specific antigen/ESA, aldehyde dehydrogenase/ALDH, CD133 and CXCR4 [150, 151]. Gemcitabine-resistant pancreatic cancer cells show higher expression of these stem cell markers [111, 113]. Sonic hedgehog (SHH), mTOR, c-Met, and Nodal/Activin signaling pathways have been implicated in pancreatic cancer cell stemness and associated drug resistance. Moreover, pharmacological inhibition of these pathways reduced cell stemness and reversed the gemcitabine-resistance [152–154]. Another feature of the cancer stem cell phenotype is EMT. Recent studies revealed that activated Notch signaling can cause drug resistance of pancreatic cancer through induction of epithelial-mesenchymal transition (EMT) [114, 143], and EMT and pancreatic tumor-initiating CD44 positive CSCs were inhibited by γ secretase inhibitors [155].

Another feature of chemoresistant CSCs is that they can contribute to tumor recurrence. Yes-activated protein 1 (Yap1) seems to be a key driver of recurrence and progression of pancreatic cancer [156]. Yap1, a protein involved in regulation of cell proliferation and apoptosis in the Hippo pathway, enables bypass of oncogenic Kras addiction in pancreatic cancer and drives anchorage-independent growth of tumor cells [156]. Yap1 can be negatively-regulated through phosphorylation by the tumor suppressor kinase Lats [157–161]. Inactivation of Lats and the Hippo tumour suppressor pathway by integrin-linked kinase (ILK) can lead to upregulation of Yap1 signaling [162]. Inhibition of Yap1 function either directly or by reactivation of Lats or targeting of ILK may be of benefit not only for treatment of metastatic tumors, but also for blocking relapse.

### *4.4 Drug Resistance Caused by Desmoplasia*

The crosstalk of pre-neoplastic or tumor cells with the immune system of the host can lead to a tumor microenvironment that is high in stroma, fibrosis and immune cells and poorly vascularized [117]. This stromal barrier prevents tumor-targeting compounds from reaching their target cells. Using animal models that mimic human tumors, efforts were made to chemically-deplete stromal tissue in order for gemcitabine to better access tumor cells. Specifically, inhibition of the SHH signaling pathway led to a transient increase in intratumoral vascular density and intratumoral enrichment of gemcitabine [117]. However it should be noted that targeting the abnormal vasculature to increase drug delivery, at the same time also increases nutrient supply to the tumors, and may have adverse effects than intended.

Secreted Protein and Rich in Cysteine (SPARC), a matricellular glycoprotein modulates diverse biological processes including extracellular matrix remodeling. In pancreatic cancer, SPARC mainly is expressed by stromal fibroblasts rather than cancer cells [163]. In addition to blockade of angiogenesis, exogenous SPARC enhances tumor stroma formation but at the same time abrogates the activation of stromal fibroblasts [164]. Recently, it has been reported that pancreatic cancer patients with low levels of SPARC show better response to gemcitabine than these with high levels, suggesting that SPARC is a prognostic marker to predict the treatment outcome of pancreatic cancer patients [165, 166].

## 5 Summary

Early diagnosis and early targeting are key for successful intervention of pancreatic cancer. Detecting patients with progressing precancerous lesions i.e. at PanIN3 stage requires reliable markers. Taking into consideration the development of feasible and noninvasive tests in the clinic, detection of circulating tumor cells using blood samples remains a first choice. Other possibilities are to determine levels of cell-free circulating tumor DNA or characteristic miRNA profiles. In addition to identifying early detection methods, more effective targeting strategies need to be developed to prolong the lifespan of pancreatic cancer patients. These include novel combinatorial use of chemotherapeutic drugs with drugs that target desmoplasia and/or chemoresistance.

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# Microbiota and Chronic Inflammation as Targets for Colorectal Cancer Prevention

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## 1 Introduction

Colorectal cancer (CRC) is a common and lethal disease (the third cancer for prevalence in men, 746,000 cases, 10.0 % of the total and the second in women; 614,000 cases, 9.2 % of the total) which is influenced by different factors, both environmental and genetic [1]. In the last few years inflammation and changes in the microbiota have emerged as important agents in the large bowel carcinogenesis.

We will discuss the core of basic knowledge in the field and we will propose new prevention approaches based on this emerging scenario.

## 2 New Issues in Chemopreventive Studies in Colorectal Cancer Stratifying by Genetic Risk

Approximately 20–25 % patients have a family history of the disease meanwhile in another 5 % CRC arises within an hereditary syndrome such as Familial Adenomatous Polyposis (FAP) caused by germ-line mutations in the APC gene, located on chromosome 5q21, and Lynch Syndrome (LS) also named Hereditary Non-Polyposis Colorectal Cancer (HNPCC) caused by germ-line mutations in DNA mismatch repair (MMR) genes [2]. Other less common hereditary diseases include MAP (MYH), Peutz-Jeghers (STK11), Cowden syndrome (PTEN) (Table 1).

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**Table 1** Hereditary syndromes

| Syndrome | Gene | Hereditary pattern |
|---|---|---|
| Hereditary non-poliposis colorectal cancer (HNPCC) | MLH1, MSH2, MSH6, MLH3, MSH3, PMS2 | Dominant |
| | EP-Cam | |
| Turcot syndrome (TS) | MMR, APC | Dominant or recessive |
| Familial Adenomatous Polyposis (FAP) | APC | Dominant |
| MUTYH-associated polyposis (MAP) | MUTYH | Recessive |
| Peutz-Jeghers syndrome (PIS) | STK11/LKB1 | Dominant |
| PTEN hamartoma tumors syndrome ( PHTS) | PTEN | Dominant |
| Juvanile polyposis syndrome ( JPS) | SMAD4-BMPR1A | Dominant |
| Polymerase Profreading-Associated Polyposis (PPAP) | POLD1-POLE | Dominant |

Major medical organizations and scientific societies indicate, as the mainstay preventive approaches for CRC, periodical screening by endoscopy and stool blood screen.

Nevertheless, several protective factors have been identified, generating interest in cancer chemoprevention.

CRC chemoprevention can be provided by the chronic administration of drugs or biological supplement (NSAIDs, estrogens, folic acid, and calcium) in order to reduce or delay the development of malignancy by preventing a variety of molecular events in tumor initiation, promotion and progression. Some of the protective agents include the Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) since, according to some researches, NSAIDs inhibit colorectal carcinogenesis. This information is derived from different sources based on epidemiologic data, intervention trials and, more recently, randomized controlled trials of NSAIDs in patients with familial polyposis, in particular Aspirin and selective COX-2 inhibitors. Aspirin is known to inhibit the expression and the activity of cyclooxygenase enzymes. In humans, there are three isoforms of the COX enzyme: COX-1 is present in all tissues and it is involved in the maintenance of the gastric mucosa integrity as well as platelet aggregation. COX-2 is involved in both inflammatory processes and carcinogenesis and it is known to be up-regulated in CRC and adenomas [3, 4] while COX-3 function is still unknown.

Despite the mechanisms of action of Aspirin and NSAIDs as chemo-preventive agents are unclear, they seem to act by decreasing prostaglandins and their derivatives (prostacyclins and thromboxanes), inhibiting cell proliferation and increasing the level of arachidonic acid, finally inducing apoptotic cell death (via COX-dependent mechanism). Another mechanism independent from COX inhibition was considered, which involves different pathways like NFκB, the peroxisome-γ proliferator-activated receptor (PPAR γ) and its ligands and could also interfere with angiogenesis [5, 6].

About 75 % of CRCs overexpress COX-2. Chan et al. reported that Aspirin is able to reduce the risk of CRCs that overexpress COX-2 but not the risk of CRCs with weak or absent expression of COX-2 [7].

The final hypothesis is that Aspirin can be used as a preventive treatment when COX-2 pathway is involved in cancer onset. More recently it has been hypothesized that Aspirin might have a direct effect on carcinogenetic events linked to PIK3/CA and on BRAF mutation. The PI3kinase mutation in colon cancer is probably involved in colon rectal carcinogenesis according to some reports on the interactions between PI3kinase and COX-2 pathways [8, 9]. Studies demonstrated that polyps size and number decreased in FAP patients with the assumption of Celecoxib, a specific COX-2 inhibitor, and Sulindac, a nonspecific COX-2 inhibitor. This means that chemoprevention agents can play an effective role in the treatment of this disorder although, at now, FDA didn't approve these drugs for chemoprevention in FAP [10, 11].

Lynch PM in a trial enrolling pediatric individuals old between 10 and 14 years demonstrated the safety and efficacy of the administration of Celecoxib for a 3-month period at all dose levels [12]. This study showed that the adenomatous polyp burden was reduced by a 16 mg/kg/day dose, with subsequent indication of 400 mg twice daily in adults.

These results can be considered to resemble the findings previously gained through the treatment with Celecoxib, although there is not a direct comparison within the randomized trial.

At present there is not a general agreement about the use of Cox-2 inhibitors in the clinical practice. Giardiello and Nugent KP in two different trials showed that Sundilac in FAP patients did not have significant benefit [11, 13].

Indeed, these trials did not meet clinically relevant endpoints as the incidence, the number or the size of polyps, when COX-2-inhibitors were compared to placebo over 4 years of treatment. On the other hand, as a result of a similar study performed on 32 patients with FAP, a daily 400 mg dose of celecoxib orally administered was effective in reducing, although not eliminating, the number of duodenal polyps after 6-months of treatment.

The same dose, assumed twice daily, gave statistically relevant results and effects only in those patients who had more than 5 % of duodenum involved with polyps at baseline [14].

Although Celecoxib appears to play an important role in the treatment of this kind of disorder, relevant side-effects have been registered and mainly consisted in cardiac events.

This means that the safety for long-term use of these agents is not proven for FAP patients as well as for general population even considering months-long exposure. Moreover, since all these trials last no more than 6 months, it is not possible to gain further clinical information about cardiac events in FAP patients who are treated with COX-2 inhibitors on a long-term basis.

Evidence of Aspirin role in chemoprevention in HNPCC derived from double-blind, placebo-controlled, randomized trial (CAPP2): 861 patients get involved in

this trial and they have been administered 600 mg/day Aspirin, Aspirin placebo, 30 g/day resistant starch or starch placebo for up to 4 years.

After 55.7 months, (range 1–128) 53 primary CRCs developed in 48 individuals: 18 of 427 belonged to the group treated with Aspirin and 30 of 434 to the placebo group. HR for CRC was 0.63 (95 % CI, 0.35–1.13; P = .12) in intention-to-treat analysis. The protective role of Aspirin in the development of colorectal cancer was recognized after 2 years of treatment [HR of 0.41 (0.19–0.86; P = .02; IRR of 0.37 (0.18–0.78; P = .008)] and in all HNPCC cancers related (such as endometrial, ovarian, pancreatic, small bowel, gall bladder, ureter, stomach, kidney, and brain cancer; HR, 0.65; 95 % CI, 0.42–1.00; P = .05) [15].

No difference in adverse events was reported between Aspirin and placebo group.

The final result of these trials was that a 600 mg of Aspirin per day, for a mean of 25 months reduces cancer incidence in HNPCC patients although the frequency of surveillance studies at the various centers have not been standardized, flawing the study interpretation.

However the same authors had conducted an earlier trial on 746 HNPCC patients that didn't achieve the same results [16].

In conclusion, several trials have been performed to validate the chemopreventive role of these drugs but final data are still discordant. Indeed the potential efficacy of chemoprevention is reduced by side-effects (for example gastrointestinal and cardiovascular) [17–19]. Therefore it is necessary, in order to assess the risk/benefit ratio, that these drugs might be of benefit only for specific individual subgroups which remain still undefined (Table 2).

**Table 2** Clinical trials

| Author | Year | No. pts | Type of study | Arms | Dose | Syn drome | End point |
|---|---|---|---|---|---|---|---|
| Philips et al. | 2001 | 83 | Randomised Double blind | Celecoxib vs Placebo | 100 mg or 400 mg twice daily | FAP | Reduction duodenal polyposis |
| Giardiello et al. | 2002 | 41 | Randomised Double blind | Sulindac vs Placebo | 75 mg or 150 mg twice a day | FAP | Mean n° or size of polyposis |
| Steinbach et al. | 2000 | 77 | Randomised Double blind | Celecoxib vs Placebo | 100 mg or 400 mg twice daily | FAP | Mean n° or size of polyposis |
| Burn et al. | 2008 | 1071 | Randomised Double blind | Aspirin vs Placebo | 600 mg/ die | HNPCC | Development of Colorectal Cancer |
| Burn et al. | 2011 | 861 | Randomised Double blind | Aspirin vs Placebo | 600 mg/ die | HNPCC | Development of Colorectal Cancer |

## 3 The CRC Microbiota

The human body is colonized by a huge number of microbes, including not bacteria only, but eukaryotes and viruses too. It is estimated that microbial species can play a key role in the pathogenesis of 15 % of all cancers. The so-called gut microbiota is a huge and complex population that influences positively and negatively host health.

In the human colon, mammalian cells and microbiota cells (bacteria, viruses, unicellular eukaryotes) live together. It is known that microbiota develops in the first 2 years from the birth and remains stable for all the individual's life. 90 % of this gut microbiota cells are bacteria: gram negative and gram positive. The positive role of microbiota in the bowel is basically the homeostasis maintenance through immune development, prevention of pathological colonization, processing diet and drug metabolites and releasing nutrient and goods from food. However, gut microbiota can produce detrimental effects. In fact a percentage of sporadic and hereditary CRCs are considered to depend from microbes resident in the bowel [20].

There are many and known condition of bacteria and viruses that are direct cause of tumorigenesis. Gastric cancer (referred carcinoma and MALT lymphoma) is linked to *Helicobacter pylori* infection in linear way. Every year 660,000 new gastric cancer diagnoses are H. pylori-related. However this does not mean that all individuals with gastric H. pylori infection eventually develop cancer but that they are at increased risk dependent from genetic diversity, specific interactions between host, bacteria and microenvironment. The mechanism through which H. pylori induces gastric cancer development is well known (NF-kB secretion IL8 mediated through CagA and VaxA interaction with gastric epithelium) and it is clear that by direct inhibition, through antimicrobial therapy against H. pylori, it is possible to reduce gastric cancer incidence. Indeed, this last evidence is the clear demonstration that the pathogenic correlation between gastric cancer and H. pylori is true and that this bacteria is the strongest risk factor for gastric cancer [20].

Another known example of correlation between microorganism and cancer in human body is represented by HCV (hepatitis C virus) and HCC (hepatocellular carcinoma). There is clear evidence that HCV infection increases the risk to develop HCC (17-fold in HCV-infected patients compared to HCV-negative patients). The mechanism through HCV infection causes HCC depends on inflammation, fibrosis and direct cell transformation. The HCV liver infection and detection of HCV at early stage can reduce HCC incidence.

The burden of resident bacteria in large intestine is more of 12-fold higher than in small intestine. This correlates with the high occurrence of CRC in large intestine as compared to the rarity of tumors occurring in small intestine and might be explained by the hypothesis that CRC is actually induced by bacteria. Many recent findings indicate a bacterial driver-passenger model for CRC. Bacteria implicated in

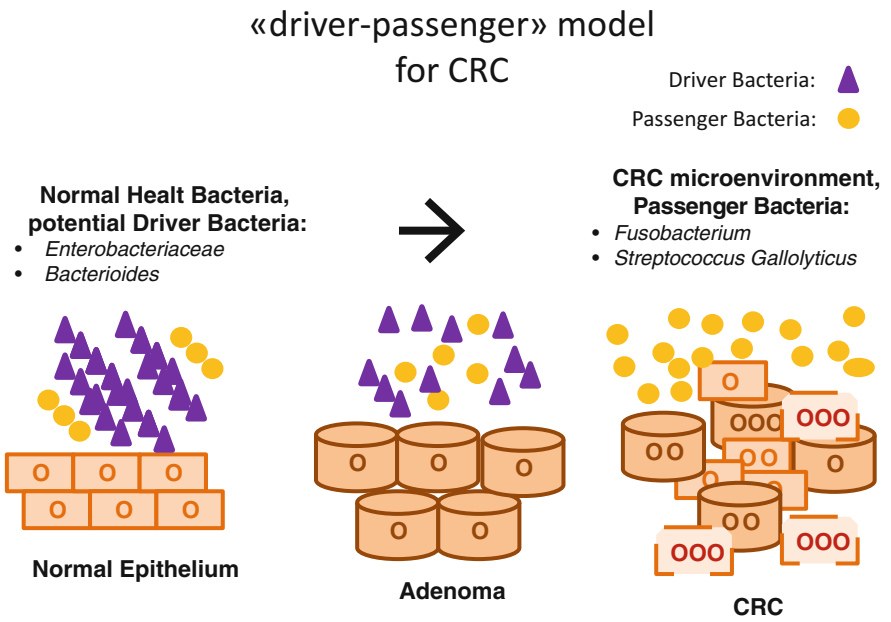


**Fig. 1** A bacterial driver–passenger model for colorectal cancer (CRC) development. According to this hypothesis driver bacteria initiate the carcinogenetic process by Damaging DNA of epithelial cells. In the subsequent phases of the carcinogenetic process, opportunistic bacteria (passenger) can overgrowth the driver bacteria and be found linked to the tumor tissue

carcinogenesis play two different roles: "drivers bacteria" defined as bacteria that potentially can initiate CRC development and "passenger bacteria" as bacteria that are found only in the biopsy of patient who have advanced CRC [20] (Fig. 1).

The CRC incidence is high in developed country and it can readily be due to the lifestyle and in particular to different dietetic habits. Indeed a diet rich of red meat and fat is related to common diseases like diabetes, cardiovascular diseases and obesity which are known to increase the risk of CRC and appears to promote inflammation, while a diet rich in fiber can exert a protective effect from CRC. A fiber-rich diet produces short-chain fatty acids like acetate, butyrate and propionate that have anti-angiogenetic and anti-tumorigenic properties that correlate with decreased incidence of CRC. On the other hand, products from meat, fat and sugar from diet like phenols, ammonia and other nitrogen are well known promoters of carcinogenesis. It is becoming clear that cancer onset is not only dependent from the diet but from the resident microbiota in bowel; from this evidence emerges the concept that diet habits and microbiota have a complex and crucial interplay. The role of inflammation in CRC development is demonstrated by high incidence of CRC in patients with IBDs (chronic colon inflammation can induce gut microbe alterations and it can promotes carcinogenesis) than in unaffected individuals and by the protective role exerted by FANS's which antagonize the polyp growth and progression to cancer (see below).

This point is crucial for understanding why alterations in composition, distribution and metabolism of gut microbes can promote inflammation, dysplasia and finally cancer [21].

The role of gut microbiota in host health is an expanding area of investigation not only in cancer but also in several human diseases. The comparison between gut microbiota in patients with adenomas and patient without adenomas demonstrated in fact a clearly different scenario [22]. Grivennikov et al. provided evidence that CRC might develop from combined effect of mucosa cell mutations and microbes in clinical and preclinical studies. Genetic mutation (like APC loss of function and Beta-catenin activation) causes the loss of gap-junction protein. This condition correlates with interaction between bowel microbiota products and resident myeloid cells with production of IL-23. IL-23 stimulates the lymphocytes to produce a TH17 cells response, that is related with poor prognosis in CRC patient, while TH1 cytotoxic response is related instead to a favorable outcome. The TH17 response induces STAT activation that promotes cell survival and proliferation. It is not clear at the present why some T-cells produce IL17 and some others produce IL1 after IL23 stimulus, but the microbiota role in this different functional response appears to be presently underscored.

The microbiota role in colon cancer development appears highly relevant in patient carriers of germ-line deleterious mutations like B-catenin and APC, but also in "sporadic" CRC tumors. The damage in bowel mucosa can promote the interaction between gut microbiota and inflammatory cells for promoting IL 23-mediated TH17 or TH1 [21].

There is a growing interest for modulating, changing and in some cases suppressing gut microbes. There is an emerging interest, from in vitro preclinical study, for probiotics. A recent study provided evidence that probiotics could have a role in antagonizing CRC development. This negative modulation is possible through many pathways that are estimated in different preclinical models although clinical evidence is based only on few studies. The most used probiotic in clinical trials is Lactobacillus Johnsonii that is demonstrated to reduce Enterobacters that produce phenol that stimulates reactive oxygen species (ROS) generation. ROS induce inflammation and promote CRC development [23]. Another clinical trial demonstrates that a 2 years treatment with Lactobacillus Casei and a fiber-rich diet reduces CRC development in a high risk population [24]. The probiotics are defined as "microorganisms that can produce benefit to the host" and that could prevent the CRC development through many different mechanisms: in part on bacterial gut (competition for bowel nutrients, inhibition of bacterial mucosal adherence, impermeability protection and control of bacterial translocation from bowel to the blood) and in part on immune system (stimulation of host immunity, macrophage activation).

Below are described the most important bacterial species implicated in CRC:

1. *Escherichia coli* is often found in CRC patients' biopsy. E. coli is responsible for the synthesis of colibactin, a genotoxic compound that is supposed to play a role in CRC. Preclinical studies (xenograft mouse model) demonstrated that the contact between colibactin produced by E. coli and bowel cells increases tumor

growth. In particular colibactin stimulates cell senescence through alterations of p53 in a post–translation mechanism as SUMOylation. At the basis of this process there is miR-20a-5p which targets SENP1 (sentrin specifc peptidase 1), which regulates the post-translation (SUMOylation) process on p53. These data indicate that in the presence of E. Coli there is cellular senescence and production of growth factors [25]. Moreover E. Coli doesn't allow DNA mismatch repair through downregulation of different proteins; this mechanism can be involved in the promotion of CRC.

2. *Fusobacterium nucleatum* and *Enterobacteriaceae* are implicated in CRC. A clinical observational study found, by qPCR in samples from CRC, ribosomal RNA gene derived by Fusobacterium and Enterobacteriaceae. This observational study compares the qPCR bacterial gene analysis between CRC patient and control patient with the evidence of different bowel microbiota composition. In this study it is also demonstrated, in fecal samples, a correlation between CRC and *Methanobacteriales* and *Methanobrevibacterium*. These findings indicate that the resident microbes are different in normal and affected patients and in affected patients in different stages of disease. The different gut microbe composition in CRC patient biopsies provides evidence than "disbiosis" is at the basis of CRC [26].
3. *Streptococcus bovis/gallolyticus* is implicated in CRC. Some CRC patients have a history of endocarditis mediated by streptococcus. It can be demonstrated that these patients (affected by Streptococcus endocarditis) could have increased risk to develop CRC with respect to individuals not affected by endocarditis.
4. In ApcMin/+ mice (mice in which there is a germline APC mutation and therefore at high risk to develop adenoma and then cancer) is demonstrated that *Bacteroides fragilis*, a common intestinal commensal species, induces CRC at higher frequency as compared to germ-free animals. Bacteroides fragilis is instead implicated in STAT3 (*Signal transducer and activator of transcription 3*) activation, IL-17 and IL-1 secretion, e-cadherin and β-cathenin secretion and activation pathway [26, 27]. Wu and colleagues found that animals exposed to this bacteria, compared to the controls, had a greater number of aberrant crypt foci (ACFs) and tumors and hypothesized that this effect could be reduced by using anti-IL-17 antibody. Most probably the activation of regulatory T cells, together with the overexpression of TH-17 cells in the colonic mucosa induced by Bacteroides leads to the development of CRC. This is in agreement with the finding of an increased inflammatory and immune cell infiltration in normal mucosa of patients with cancer detected at colonscopy compared to healthy individuals [28]. However, if this is the direct cause of cancer of the colon or the result of confounding factors from the environment still remains to be explored [29].
5. *Streptococcus gallolyticus* and *Fusobacterium* could be implicated like bacteria in carcinogenesis and CRC development; in fact they are found in biopsy of patients affected by precancerous adenomatous polyps if compared to non affected individuals [26].

**Table 3** Bacterial and CRC: correlation

| Microbe | Carcinogenetic mechanism |
|---|---|
| *Bacteroides fragilis* | Stat3 Activation; IL1 and IL17 secretion and stimulation; E-cadherin and Beta Cathenin pathway activation |
| *Escherichia coli* | P53 downregulation and cellular senescence through colibactin production |
| *Enterococco faecalis* | ROS production |
| *Bacteroides vulgates* | NFkB and MYD 88 (Myeloid differentiation response gene 88) activation |
| *Streptococcus bovis* | Increased proliferation through IL8 production |
| *Clostridium butyricium* | Bacterial Genes are found in CRC patient tissue through qT-PCR |
| *Mitsuokella multiacida* | |
| *Streptococcus gallolyticus* | |
| *Fusobacterium nucleatum* | |

6. *Enterococcus faecalis*, through stimulation of ROS induces carcinogenesis in susceptible patients. The ROS presence stimulate inflammation in the bowel and in patient with APC mutation this condition causes a pathogenic interaction between bacterial and immune system and drives to carcinogenesis induction [25] (Table 3).

The previously described bacteria are CRC-related by different means and trough mechanisms not completely understood. They can depend from different external and internal body conditions. It is now a common concept that the gut microbiota has a carcinogenetic role but it has not been identified a single target which can be used in the prevention and cure of cancer. It is now important to understand how it could be possible to reduce CRC risk through probiotics and prebiotics. It has to be considered that the microbiota can be modulated not only for the prevention and the cure of cancer but also for potentiating drug efficacy and reducing drug toxicity. All cytotoxic drugs cause different and in some case dangerous adverse events. For example irinotecan, an anti-CRC drug which acts as topoisomerase-1 inhibitor, causes immune suppression and diarrhea. This adverse event is related to the carboxylesteration and glucoronidase activity of irinotecan (prodrug) in active form. In some patients during irinotecan treatment, a severe and refractory diarrhea occurs that sometimes can lead to treatment withdrawal. This dangerous and severe type of diarrhea can be related to different gut microbiota which includes some bacterial that transform irinotecan in its active form in the bowel causing diarrhea. From these findings, ongoing trials are investigating drugs that can block bacterial glucoronidase for the improvement of drug efficacy and reducing drug-toxicity. This method can represents an exciting direction for microbiota-based cancer therapeutics. The bowel bacteria can contribute to chemotherapy responsiveness also by influencing immune system. For example it has been observed that oxaliplatin, an anti-CRC drug, depends for its function and efficacy on the gut microbiota-immune

system interactions. The same condition is found in the higher activity and efficacy of platinum in patients not affected by CRC; instead the chronic inflammation determined by gut microbiota stimulates the reactive oxygen compounds formation and the modulation of some enzymes with a key role for response mediated by myeloid cells. The ROS production produces high stress levels into the cancer cells and potentiates DNA damage and oxaliplatin-dependent cancer cell death. Gut microbiota represents today, for its pro and anti-carcinogenetic effect and for its role in drug efficacy and resistance mechanism, the most interesting scenario in CRC research in the last 40 years. Specific bacterial, virus and other bowel human microorganism represent now potential targets for decreasing cancer risk and modulating the efficacy, toxicity and the resistance to anticancer drugs [30].

Even if all these findings are highly suggestive for a crucial role of microenvironment in CRC, definite evidence on the underlying mechanisms is still lacking. The microenvironment is composed by gut microbiota that can be changed by diet, hormone, metabolic disease, smoke, drug and lifestyle able to promote CRC onset in sporadic and hereditary conditions.

## 4 Chronic Inflammation and Colorectal Cancer

### *4.1 Chronic Inflammation*

The last decade has witnessed a growing awareness on the role of chronic inflammation in inducing immunosuppression and cancer. Indeed, Rudolf Virchow, in nineteenth century, hypothesized that inflammation could have a role in the development and progression of cancer, identifying major susceptibility and occurrence of this disease in the sites of chronic inflammation [31–34].

Several studies showed a role of chronic inflammation in the pathogenesis of various cancers. This finding is potentially confirmed by experimental and clinical findings where the use of non-steroidal anti-inflammatory drugs (NSAIDs) has a protective role against different tumors [35, 36]. It has been recently shown in humans and animal models that intestinal microbiota and host immunity are involved in the progression of large bowel diseases [37]. Inflammatory conditions, as the inflammatory bowel diseases (IBD) Crohn's disease and ulcerative colitis, correlated with an increased risk of development of colorectal cancer (CRC) as well as inflammatory microenvironment has a clear role in breast cancer or multiple myeloma occurrence [38]. Firstly, neutrophils, lymphocytes, macrophages, dendritic cells (DCs) and myeloid derived suppressor cells (MDSCs) represent the cells attracted and recruited in site of injury in response to inflammation; while inflammatory factors including cytokines, chemokines, transcription factors and microRNAs (miRNAs) promote carcinogenesis in CRC [39].

Moreover, the macrophages play a leading role in the interaction between cancer and inflammation because they are able to:

- induce cell growth and survival, including, therefore, the tumor cells, through secretion of bFGF, PDGF, EGF, IL6, TNF;
- promote angiogenesis through the expression of angiogenetic molecules such as VEGF and MMPs, CXCL1, CXCL8, HIF1-alpha;
- determine invasion and metastasis: chemokines, MMPs
- induce mutations by producing superoxide and peroxynitrite;
- inhibit T-cell response via production of IL10, TGF-beta.

Another important component of the tumor microenvironment are lymphocytes.

Several observations suggest that Th17 lymphocytes are involved in pathogenesis of different autoimmune diseases and pathologic inflammatory states. Their activation is related to poor prognosis in CRC patient. Recently, there is evidence that Th17 cells contribute to the pathogenesis of Inflammatory Bowel Diseases (IBDs) and CRC, but their specific role in both diseases is unknown [40]. In particular, IL17 is a cytokine that is considered to play the main role. Indeed, IL17 binding to its receptor, that is ubiquitous, induces the activation of the NF-kB and signaling of JUN amino-terminal kinase (JNK) in a manner dependent on the Tumor necrosis Factor Receptor (TNFR) associated with TNFR-6. It has been demonstrated that IL17 is secreted by CD8 + T cells, γδ-T cells and neutrophils, but this effect remains still undefined. The last two cell species are rapidly recruited, in the first phase of the response to infection, so probably regulate the innate inflammatory response as the infiltration of neutrophils and macrophages in infected tissues. However, the loss of inflammatory response control can become an important cofactor in the pathogenesis of many chronic human diseases, including cancer. NF-kB is a basic pathway of inflammation and appears to play a crucial role in tumor promotion [41].

Moreover, toll like receptor 4 (TLR4) is part of what immunologists call the "innate immune system" which acts as a first line of defense against harmful substances and plays a key role in protecting against the development of tumors and chronic inflammation. TLR4 induces a survival pathway on epithelial intestinal cells by the cellular signaling which leads to nuclear translocation of NF-kB.

In conclusion, literature data agreed that inflammation is a critical component of tumor progression. Many cancers arise from sites of infection and inflammation. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant to the carcinogenetic process, fostering proliferation, survival and cellular migration. In addition, cancer cells utilize some of the signaling molecules of the innate immune system, such as selectines, chemokines and their receptors for invasion, migration and metastasis. For all these reasons, research is focused to development of new anti-inflammatory therapeutic approaches for cancer prevention. In support to the inflammation role in tumor development, it has been found an increase in C-reactive protein (CRP) serum levels in CRC patients. We have described that the discovery that the enzyme COX-2 is overexpressed in colorectal cancer, gastric and intestinal metaplasia, Barrett's

esophagus, liver cancer, pancreatic cancer, endometrial cancer and skin, has led to the finding that drugs Celecoxib which inhibit inflammation indeed antagonize tumor occurrence (see Sect. 2).

### *4.2 miRNA and Colorectal Cancer*

Until the discovery of epigenetic regulation of carcinogenesis, it was believed that a deregulation of oncogenes and tumor suppressor genes was the exclusive basis of tumor development. The carcinogenetic process is indeed often caused by genetic defects, such as point mutations, insertions/deletions, and chromosomal rearrangements that cause overexpression of oncogenic protein or down-regulation of tumor suppressors. It is now however becoming clear that other events that cause deregulation of crucial gene expression are the epigenetic modifications, including DNA methylation. A new frontier for understanding the mechanisms of carcinogenesis was offered by the discovery of microRNAs (miRNAs). The altered expression of miRNAs is correlated to a variety of cancers, because it has been found that these molecules may function as oncogenes or as tumor suppressor genes. miRNAs are small (19–22 nucleotides) non-coding RNA molecules that regulate 20–30 % gene expression at the post-transcriptional level by base-pairing to specific messenger RNAs (mRNAs) [42], promoting their degradation or suppressing translation. miRNAs can act as inflammatory mediators, oncogenes or tumor suppressors in different cellular environments. miRNAs also serve as biomarkers and therapeutic targets in CRC. The risk of CRC is also influenced by miRNA polymorphisms and binding sites. Their functions as diagnostic biomarkers or prognostic classifiers has been demonstrated.

One of the miRNAs identified as a potent tumor suppressor in the CRC and inhibitor of COX-2 mRNA translation, whose expression is reduced in tumor cell lines of CRC and in tumor tissues of colon is miR-101 [43, 44]. Indeed, there is a reduced miR-101 expression in various types of cancer, such as colorectal, gastric, thyroid, lung, liver, pancreatic, breast and prostate cancer, and translational inhibition is indeed produced on different oncogenes. Strillacci et al. (2012) showed a direct regulation of β-catenin gene by miR-101 [44]. Finally, in the context of inflammation it has been proposed a model of regulated miR-101 expression in which pro-inflammatory agents (LPS) are able to repress the expression of the miRNA, while the anti-inflammatory agents (NSAIDs) upregulate its expression.

## 5 New Directions

The several points discussed in this review share the same strong conceptual basis: inflammatory status is able to influence microenvironment and tumor development.

Nevertheless, only little evidence has been achieved by studies aimed to consider the whole scenario involving all these factors. Indeed, Literature misses epidemiological studies in which a chemoprevention treatment was evaluated both for classic efficacy criteria (number and size of polyps) and for possible variations on the gut microbiota. This goal is a necessary step to understand if modulation of inflammation can interact positively on the intestinal flora, prospecting a potential powerful effect based on the combinatory activity. A further exploratory study to be designed on this topic, could be the investigation of a "molecular portrait of microbiota in stool sample" in which several information could be achieved and put in correlation, including blood stool occult test (already considered in screening programs) and possibly the search for tumor DNA. All this information might be of precipual interest for the major goal of early diagnosis of CRC, even in the developmental process. It would be interesting to see if these portraits change in the pre different stages of the disease or according to medical treatment or in presence of different molecular subtypes of disease or in link with the expression of several biomarkers and cytokines as previously reported. If these hypotheses are confirmed and validated in prospective case-control studies, it could be possible to consider this information with diagnostic and prognostic purpose, as well as recently described for liquid biopsy, currently "on the stage" for other diseases.

The role played by microbiota and inflammation in colorectal carcinogenesis adds new complexity but also novel opportunities for a tailored approach to prevention and even treatment of this disease. The clear interconnection among microbiota and inflammatory status opens the way to novel preventive approaches which might be aimed to avoid the development of a permissive microenvironment by modifying the microbiota landscape. This approach might be of major value in order to avoid the risk of toxicity of long term treatment with anti-inflammatory drugs as COX1 inhibitors, as detailed in the article.

It should be important to gain novel information on diet habits and microbiota in order to set rational nutrition-based intervention at population level.

At this aim it is important to consider that microbiota appears to play a role in a variety of different diseases and it is therefore important to have a multidisciplinary vision in order to design multi-targeted multi-preventive interventions.

The novel molecular sequencing technologies like Next generation sequencing (NGS) and the integrative genomics approaches which allow to integrate data from NGS with molecular profiling technologies offer the basic platform to identify critical nodes comparing normal mucosa to pre-neoplastic and tumor lesions allowing the understanding of interaction among all the participant to the colon rectum landscape, including mucosal and stromal cells, inflammation-related cell populations and the microbiota.

Such information, which might be integrated by studies in novel animal models will be the basis for the identification of surrogate endpoints of activity, to select individuals for more or less intensive intervention.

It is also important to consider that the identification of low and medium penetrance determinants of genetic risk with the modern vision of "molecular portraits"

can provide an important tool for the identification of individuals for preventive approaches and finely tune their risk/benefit and cost/effectiveness.

The modern view of inflammation and microbiota have indeed produced a paradigm change in our understanding of CRC pathobiology and offer the rationale for new generation preventive intervention.

It is now time for a coordinated effort for the complex but achievable goal of CRC prevention.

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# Challenges in Multiple Myeloma Chemoprevention: Potential Role of Natural, Synthetic and Endogenous Molecules

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## 1 Introduction

Carcinogenesis is a multistep process characterized by the accumulation of molecular aberrations leading to transformation and uncontrolled cell growth. Several factors within the tumor microenvironment may contribute to carcinogenesis, which generally requires long latency for development of invasive tumors [1]. At least three stages, namely initiation, promotion and progression, may concur to carcinogenesis. Initiation involves DNA damage, which is caused by carcinogens, and this process is often rapid and irreversible. Conversely, promotion is a relatively slow and reversible process that involves epigenetic changes. The last stage is the progression, characterized by the acquisition of a fully malignant phenotype [2].

A wealth of studies has recently demonstrated the possibility to control the carcinogenetic steps, especially in those cancers characterized by long latency. Indeed, multiple myeloma (MM) represents the prototype of these malignancies given the existence of well-known pre-malignant conditions which include monoclonal gammopathy of undetermined significance (MGUS), indolent multiple myeloma (IMM) and smoldering multiple myeloma (SMM). MM, which can also occur *de novo*, is often a subsequent, late-stage of this progression, characterized by higher infiltration and accumulation of malignant plasma cells in the bone marrow (BM) and

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monoclonal protein in the blood [3]. It is the second most common hematologic malignancy after non-Hodgkin lymphoma, accounting for more than 10 % of all hematologic cancers and 2 % of annual cancer-related deaths [4]. MM cells home to the BM, which offers a survival and drug-resistance framework by direct interaction of tumor cells with bone marrow stromal cells (BMSC) and extracellular matrix (ECM) components [5, 6]. The aberrant antibodies that are produced contribute to the deregulated humoral immunity in MM patients [7]. Approximately, 1 % of patients with MGUS develop MM each year [6]. On the basis of cytogenetic abnormalities, MM can be classified as hyperdiploid or non-hyperdiploid MM [8, 9]. The first one is characterized by trisomies of odd numbered chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, while non-hyperdiploid MMs contain various translocations involving the immunoglobulin heavy chain (IgH) locus [10]. Current standard treatments for MM are high-dose chemotherapy and autologous stem cell transplantation for younger eligible patients [11]. However, although novel research platforms are emerging [12–15] and new agents reached advanced phases of preclinical or clinical investigation, current experience indicates that a big portion of high-risk patients does not benefit even from double or triple combinations of new agents, including bortezomib (Velcade), thalidomide (Thalomide), and lenalidomide (Revlimid), alone or in association with conventional cytotoxic agents [12–14, 16–19]. With improved high-dose chemotherapy supplemented with autologous stem cell therapy, the median survival rate of MM patients has increased to 5–7 years [20]. In contrast to MM patients who receive chemotherapy, patients with MGUS, SMM and IMM have stable disease and do not undergo to chemotherapy. However, patients who experience MGUS with paraproteins ≥1.5 g/dL, SMM or IMM are at high risk of developing a clinically active MM. Such patients appear therefore good candidates for novel therapeutic strategies aimed at inhibiting or preventing the development of a clinically active disease [21].

Over the last decade, accumulating evidence clearly indicates that multiple pro-inflammatory signaling pathways play a pivotal role in proliferation, migration, adhesion, and survival of MM cells [22]. For example, signaling mediated by the transcription factor NF-κB is constitutively active in MM and in BMSCs, the latter producing cytokines which in turn activate NF-κB to trigger proliferation of MM cells [24]. Thus, the use of effective strategies to inhibit such pro-inflammatory signaling pathways can represent a viable therapeutic option for MM patients.

## 2 Basic Principles of Chemoprevention in MM

Chemoprevention consists in the use of non toxic agents, dietary and natural compounds as tools for controlling disease progression. Dietary constituents, along with natural products, have proven to hamper numerous signaling pathways involved in cancer development.

Several phytochemicals, plant-derived natural products, have been associated to anti-cancer properties in MM [25], due to their ability to affect a variety of pro-inflammatory signaling cascades involved in the proliferation, survival and metastasis

of MM [26–28]. Although the exact molecular mechanism of action of these components has not been fully elucidated, accumulating findings on curcumin, resveratrol, betulinic acid and many other agents targeting pro-inflammatory signaling molecules have demonstrated potential advantages for the management of MM. Moreover, it is widely acknowledged that dietary components may also affect the epigenome, and this notion has prompted a new approach in cancer prevention named "nutriepigenetics".

Epigenetics represents an emerging set of mechanisms revealing how the environment, including food and nutrition, might shape and affect the genome [29]. Major epigenetic mechanisms of gene regulation include DNA methylation, modifications of the chromatin structure by histone acetylation and methylation, and non-coding RNAs.

DNA methylation is the reaction catalyzed by DNA methyltransferases (DNMTs) which exploits S-adenosyl-L-methionine (SAM) as donor of methyl groups. Three different DNMT isoforms have been thus far characterized: DNMT3A and DNMT3B, which are responsible for *de novo* DNA methylation, and DNMT1, which accounts for replication of the DNA methylation pattern in genomic DNA [30].

The effect of DNA methylation is dependent on the location of methylation sites within a gene [31]. In general, locus-specific hypermethylation at CpG islands located in the promoter region of tumor suppressor genes are detectable in many hematologic neoplasias such as MM [32], where it is responsible for gene silencing. This event is frequent for genes or non-coding RNAs involved in cell cycle regulation, cell invasion, growth factor signaling, DNA repair and immune modulation.

Acetylation of histone tails' lysine residues affects the interaction of histones and DNA in nucleosomes. Hyperacetylation leads to a more relaxed state of chromatin which enhances accessibility of the transcription machinery; conversely, hypoacetylation results in a more compact chromatin state and generally leads to gene silencing. The balance of histone acetylation depends on the activity of two enzyme groups: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDAC proteins negatively control the acetylation state of lysine residues located on the amino-terminal tails of histone proteins, hence these proteins are generally associated with repression of transcription and reduced gene expression [33].

Overexpression of class I and class II HDAC proteins has been observed in human cancer [34], including MM [35]. Consequently, HDACs have emerged as attractive targets for both chemopreventive and therapeutic strategies [33, 34]. HDAC inhibitors (HDACi) act by promoting the retention of acetyl groups on histone tails, thus allowing a more active and open chromatin conformation [36].

A wealth of data has reported epigenetic silencing of detoxifying enzymes, tumor suppressor genes, cell cycle regulators and several other genes by promoter methylation, and modifications of histones and non-histone proteins such as p53, NF-κB or chaperone proteins by acetylation or methylation.

Since epigenetic modifications are reversible and occur early during carcinogenesis as potentially initiating events, chemopreventive agents could affect these cancer-related epigenetic alterations by influencing the activity or expression of DNMTs and HDACs.

Chemopreventive agents with reported mechanisms targeting the epigenome include micronutrients, polyphenols, genistein and soy isoflavones, curcumin,

antibiotics, pharmacological agents, modulators of histone lysine methylation and many others [37].

Compounds with known anti-tumor activity in MM will be discussed within this chapter, along with other synthetic or endogenous molecules such as non-coding RNAs (microRNAs) with a potential role in preventing and/or treating MM.

## 3 Phytochemicals

### *3.1 Curcumin*

Among phytochemicals, curcumin (diferuloylmethane), a polyphenol from the plant *Curcuma longa*, is indeed the most studied. The potential use of curcumin in cancer chemoprevention seems correlated to its pro-apoptotic, anti-proliferative, anti-oxidant and anti-angiogenic activities.

Emerging evidence indicates that curcumin suppresses a number of key cellular transduction pathways, such as phosphorylation catalyzed by protein kinases, c-jun-activated protein 1 (AP-1) activation and prostaglandin biosynthesis. Curcumin also induces the production of reactive oxygen species (ROS) and leads to apoptosis. It has been shown that curcumin possesses anti-inflammatory activity through inhibition of cyclooxygenase-2 (COX-2) gene expression. By NF-κB inactivation, curcumin downregulates the expression of various pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), interleukins (IL-1, IL-2, IL-6, IL-8, IL-12), while it activates p38 mitogen-activated protein kinases (MAPK). In addition, curcumin suppresses matrix metalloproteinase (MMPs) 2 and 9 expression, which are involved in tumor angiogenesis. Furthermore, beta-catenin (β-catenin), T-cell factor (TCF), or lymphoid enhancer factor (LEF) signaling is dampened by curcumin in many cancer cells. It has been observed that curcumin enhances Akt dephosphorylation, inhibition of anti-apoptotic protein such as B-cell lymphoma 2 and B-cell lymphoma-XL (Bcl-2, Bcl-XL) and blocks inhibitors of apoptosis (IAP). Curcumin also promotes cytochrome-c release and activation of caspase 3. Given the central role of NF-κB in MM cell survival and proliferation, authors explored this transcription factor as a target of curcumin's action. Curcumin was found to downregulate the nuclear pool of NF-κB and to suppress constitutive IkBα phosphorylation, IKK kinase activity, and expression of NF-κB-regulated gene products IkBα, Bcl-2, Bcl-xL, cyclin D1, and IL-6. Consistently, inhibition of proliferation, blockade of cells at the G1/S cell cycle phase, and induction of apoptosis was induced by curcumin in MM cells. Curcumin also increased chemosensitivity to vincristine and melphalan [23]. Notably, Sung et al. found that curcumin potentiated the apoptotic effects of thalidomide and bortezomib by downregulating constitutively active NF-κB and Akt. Moreover, the authors found that curcumin potentiated the *in vivo* antitumor effects of bortezomib in a nude mice model, and this correlated with suppression of Ki-67, CD31 and VEGF expression [38]. In a study involving 26 MGUS patients, Golombick et al. found that 4 g/day of oral curcumin decreased

paraprotein load in a selected group of patients with paraprotein levels >20 g/L, with a significative reduction of paraprotein in 50 % of the patients undergoing curcumin administration. These results indicate that curcumin warrants further investigation for the management of MGUS [39]. A number of studies investigating the safety and efficacy of curcumin has been carried out, which reveal its poor absorption and rapid elimination from the human body with no evidence of adverse effects [40]. Interestingly, novel curcumin analogues, endowed with a stronger *in vitro* anti-MM activity when compared to curcumin, have been recently synthetized [41].

## *3.2 Resveratrol*

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin found in grapes [42], which was found to exert activity against a wide variety of tumors, including MM [43]. Several mechanisms have been linked to the anti-cancer activity of this compound, among others: cell-cycle arrest, induction of apoptosis via Fas/CD95, tubulin polymerization, upregulation of p21, p53 and Bax, downregulation of survivin, cyclin D1, cyclin E, Bcl-2, Bcl-xL and IAP, activation of caspases, suppression of adhesion molecules and inhibition of angiogenesis, invasion and metastasis [43, 44]. It was shown that resveratrol overcame drug-resistance in MM cells and potentiated the apoptotic effects of bortezomib and thalidomide [43]. In addition, resveratrol inhibited in a dose-dependent manner the differentiation of monocytes in tartrate acid-resistant phosphatase (TRACP) positive multinucleated cells, and downregulated RANK and NFATc1 expression, thus reducing the osteoclastic activity; moreover, the authors found that resveratrol induced the expression of osteoblastic markers in human BM mesenchymal stem cells [45]. These results suggest that resveratrol might be potentially useful against MM-related bone disease.

## *3.3 Chaetocin*

Chaetocin, a fungal metabolite produced by *Chaetomium species* fungi with a epidithiodiketopiperazine alkaloid structure, is a selective inhibitor of SUV39 histone lysine methyltransferases targeting H3K9, whose trimethylation is generally associated with repressed chromatin. Isham et al. demonstrated that chaetocin elicits anti-MM activity by generation of oxidative stress and apoptosis induction, without affecting normal B-cells. Chaetocin displayed higher *ex vivo* antimyeloma activity and selectivity than doxorubicin and dexamethasone [46]. Moroever, the same authors found that chaetocin might represent a potential anti-MM therapeutic based upon its chemical structural similarities to the acetylated lysine residue of histones that is mimicked by HDAC inhibitors. Therefore, chaetocin appears a promising agent for further clinical investigation as anti-MM agent.

### 3.4 Guggulsterone

Guggulsterone (GS) is the major constituent of the gum resin and has two stereoisomers, E-GS (cis-GS) and Z-GS (trans-GS). GS has demonstrated anti-inflammatory, antioxidant, hypolipidemic, hypocholesterolemic and hypoglycemic activities. It has been originally described as a farnesoid X receptor antagonist. Ahn et al. [47] demonstrated that the Z but not the E stereoisomer of GS inhibited both constitutive and IL-6-induced STAT3 activation in human MM cells. GS down-regulated the expression of STAT3-regulated antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1), proliferative (cyclin D1) and angiogenic (VEGF) gene products, and this correlated with suppression of proliferation, accumulation of hypodiploid cells and induction of apoptosis.

### 3.5 Celastrol

Celastrol is one of the main active components extracted from the traditional Chinese medicine *Tripterygium wilfordi*. Celastrol effectively blocks the nuclear translocation of NF-κB p65 subunit and induces human MM cell cycle arrest and apoptosis *via* p27 upregulation and NF-κB modulation. Celastrol-induced apoptosis in MM cell lines involves activation of the caspase-3 and NF-κB pathways [48]. Moreover, celastrol potentiated the apoptosis induced by TNF and chemotherapeutic agents and inhibited invasion, both regulated by NF-κB activation. TNF treatment was accompanied by the increase of anti-apoptotic (IAP1, IAP2, Bcl-2, Bcl-XL, c-FLIP, and survivin), pro-survival (cyclin D1 and COX-2), pro-invasive (MMP-9), and pro-angiogenetic (VEGF) gene products, while celastrol treatment suppressed their expression. Celastrol was also found to inhibit the TNF-induced activation of IkBα kinase, IkBα phosphorylation, IkBα degradation, p65 nuclear translocation and phosphorylation and NF-κB-mediated target gene expression [49]. Some studies have also reported that celastrol inhibited the proliferation of MM cells regardless of their sensitivity to bortezomib or other conventional chemotherapeutic drugs. Celastrol also inhibited constitutive and IL6-induced activation of STAT3, and induced apoptosis demonstrated by an increase in cells in sub-G1 phase, an increase in the expression of pro-apoptotic proteins and activation of caspase-3 [50].

### 3.6 Thymoquinone

Thymoquinone (TQ), the major active component of the medicinal herb *Nigella sativa Linn*, has been described as a chemopreventive and chemotherapeutic compound [51]. TQ suppressed constitutive and IL-6-induced STAT3 activation in

human MM cells along with inhibition of c-Src and JAK2 kinases. TQ also downregulated the expression of STAT3-regulated gene products; it also inhibited MM cell proliferation, promoted accumulation of cells in sub-G1 phase and apoptosis, and significantly strengthened apoptosis elicited by thalidomide and bortezomib in MM cells [52]. TQ treatment also inhibited chemotaxis and invasion induced by CXCL12 in MM cells [53] and significantly downregulated CXCR4 expression and CXCL12-mediated CXCR4/CD45 association in MM cells. TQ also induced the re-localization of cytoplasmic Fas/CD95 to the membrane of MM cells and increased CD95-mediated apoptosis [54].

## *3.7 Vitamin D*

Vitamin D is a fat-soluble prohormone synthesized in response to sunlight, present in two main forms: vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). Vitamin $D_3$ is formed in the skin, after exposure to ultraviolet B radiation, from 7-dehydrocholesterol that resides in the cell membranes; vitamin D2 is obtained through irradiation of ergosterol in plants, and enters circulation through diet (i.e., animal sources such as deep sea fatty fish, egg yolks, or liver). Experimental evidence indicates that vitamin D may reduce the risk of cancer through regulation of cellular proliferation and differentiation as well as inhibition of angiogenesis. These anticancer properties have been attributed primarily to 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] (calcitriol), the hormonal form of vitamin D. Cancer cells express specific receptors (VDR) for 1,25-dihydroxyvitamin D. When bound to the VDR, 1,25-dihydroxyvitamin D regulates the expression of genes exerting prodifferentiating, antiproliferative and antimetastatic effects on cells. Adequate vitamin D status has been linked to decreased risks of developing specific cancers, including cancers of the hematopoietic system (e.g., Hodgkin's and non-Hodgkin's lymphomas, MM). Vitamin D deficiency has been observed in MM, with 40 % of patients having vitamin D levels less than 36 nmol/L [55]. Vitamin D and its metabolites have a significant clinical role in these patients because of their interrelationship with calcium homeostasis and bone metabolism [56]. Vitamin D deficiency causes hypocalcemia, which stimulates parathyroid hormone secretion and activates a cascade of biochemical events negatively affecting bone anabolism. Vitamin D deficiency leads to decrease in intestinal calcium absorption and an increase in PTH. In addition to the role of vitamin D in the maintenance of skeletal homeostasis, studies have reported that vitamin D plays a role in the inhibition of carcinogenesis by induction of differentiation, inhibition of cellular proliferation and angiogenesis, and promotion of apoptosis [10]. Park et al. provided evidence that the 1,25-dihydroxyvitamin D3 analog EB1089 inhibits the cell growth of NCI-H929 MM cells *via* G1 cell cycle arrest and apoptosis induction by activating p38 kinase and suppressing ERK activity [14].

### 3.8 Isothiocyanates

Isothiocyanates (ITC) are dietary components present in cruciferous vegetables as broccoli, cabbage and kale, characterized by a sulfur containing functional group (N=C=S). Common isothiocyanates include: allylisothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) [57]. Isothiocyanates have been reported to prevent the initiation of carcinogenesis, as well as to inhibit the progression stage. Authors have shown that PHI, a synthetic isothiocyanate, affects the epigenome as an HDAC inhibitor causing hyperacetylation of histone H3 and also induces hypomethylation of tumor suppressor *p16* in MM cells. PHI inhibited the growth of the MM cells and induced apoptosis through disruption of the mitochondrial membrane potential; PHI also inhibited IL-6 receptor expression and VEGF production in MM cells, and reactivated p21 expression [57]. BITC and PEITC are 2 strongest proteasome inhibitors whose activity correlates with the rapid accumulation of both tumor suppressor p53 and NF-κB inhibitor, a mechanism by which ITCs inhibit growth of MM cells through promotion of cell cycle arrest at $G_2$/M phase and apoptosis triggering [58].

### 3.9 Epigallocatechin-3-gallate

Green tea has potent antioxidative properties especially the tea catechin (−) epigallocatechin-3-gallate (EGCG). The anti-tumor effects of this compound include inhibition of angiogenesis, modulation of growth factor-stimulated proliferation, suppression of oxidative damage, induction of apoptosis and/or cell-cycle arrest. EGCG induces apoptotic cell death in IL-6-dependent and -independent MM cells, without any effect on normal cells, and induces apoptosis *in vitro* and *in vivo* in a murine model of human MM. The anti-MM effects of EGCG are mediated through a 67-kDa LR1, a cell-surface receptor implicated in the interaction of MM cells with basement membrane and subsequent infiltration/migration of these cells in surrounding tissue. Treatment with EGCG induces death-associated protein kinase 2 (DAPK2) in MM cells [59]. EGCG also induces translocation to the plasma membrane of acid sphingomyelinase (aSMase), that is critical for the lipid-raft clustering and apoptotic cell death, and PKCδ (protein kinase Cδ) phosphorylation at Ser664, which is important for 67LR-mediated aSMase/ceramide signaling [60]. EGCG treatment has been also associated with elevated mRNA and protein levels of p73 and p63, two members of the p53 family described as inducers of apoptosis. Since p53 is frequently mutated in cancers, the induction of p53-like proteins by EGCG offers an alternative mechanism of growth arrest in the absence of p53 [59]. EGCG also reduces the expression of four p53-targeting miRNAs, i.e. miR-25, miR-92, miR-141 and miR-200a [61]. Moreover, due to its antioxidant effect, EGCG reduces levels of peroxiredoxin V (PrdxV), inducing apoptotic cell death [62].

### 3.10 Capsaicin

Capsaicin (CAP) is the pungent principle found in the hot red peppers and the chili peppers that has been linked with suppression of tumorigenesis through a mechanism that is not well understood. Capsaicin acts as blocker of the STAT3 activation pathway. The activation of Janus-activated kinase 1 and c-Src, implicated in STAT3 activation, was also inhibited by the vanilloid, with no effect on extracellular signal-regulated kinase 1/2 activation. Capsaicin downregulates the expression of the STAT3-regulated gene products, such as cyclin D1, Bcl-2, Bcl-xL, survivin, and VEGF, and induces the accumulation of cells in G(1) phase and caspases activation [63].

### 3.11 Baicalein

Flavonoids are a huge class of polyphenol phytochemicals found in most fruits and vegetables, and have been demonstrated to have various favorable effects on human health, such as antioxidant, anti-inflammatory, anti-viral and anti-carcinogenic properties. Scutellaria (Lamiaceae) is a genus, which includes about 350 species, whose medicinal potential is due to the flavonoids and their glycosides. Baicalein forms a major component in S. baicalensis which is known for its efficient cytotoxic activity against cancer cells [64]. Some reports demonstrated that a novel mechanism of action for Scutellaria extract and its main active flavonoids is the targeting of side population cells (putatively cancer stem cells) by modulating the expression of ABCG2 protein [65]. Moreover, baicalein dampens MM cell proliferation and migration, these effects being associated with inhibition of proliferation- related genes as β-catenin, c-myc, cyclin D1 and integrin β7 [66]. Baicalein also inhibits the phosphorylation of IkBα, followed by decreased expression of IL-6 and XIAP genes and activation of caspase-9 and caspase-3 in MM patients' plasmacells [67].

### 3.12 Betulinic Acid

Betulinic acid (BA) is a lupane-type pentacyclic triterpene [68] inducing apoptosis and blocking the autophagic flux in MM cells; the inhibition of autophagic flux also contributes to BA-mediated apoptosis of MM cells. BA treatment dose-dependently increased the accumulation of LC3-II and p62 in MM cells, thus leading to inhibition of the autophagic flux. Furthermore, BA treatment down-regulated the expression of Beclin-1, an important inducer of autophagy, in MM cells [69]. Moreover, BA inhibits constitutive activation of STAT3, Src, JAK1 and JAK2 kinases. [70]. BA also affects cell cycle by promoting cell arrest at G0/G1 phase [71].

### 3.13 Butein

Butein is a polyphenol, one of the compounds of chalcones, a class of flavonoids that are widely biosynthesized in plants. Plants containing butein have been used in Chinese traditional medicine. Recently, it has been reported that butein suppresses proliferation and triggers apoptosis in various human cancer cells *in vitro* and *in vivo* [72]. Studies indicate that this chalcone inhibited both constitutive and IL-6-inducible STAT3 activation in MM cells through the inhibition of activation of the upstream kinases c-Src, JAK1 and JAK2. Butein downregulated the expression of STAT3-regulated gene products such as Bcl-xL, Bcl-2, cyclin D1 and Mcl-1, and this led to the suppression of proliferation and induction of apoptosis [73].

Importantly, butein plays a critical role in suppressing MM-induced osteoclastogenesis by blocking NF-κB. RANKL, a member of the tumor necrosis factor superfamily, is the major mediator of bone resorption. Butein blocks MM cell-induced differentiation of macrophages to osteoclasts *via* inhibition of IκBα kinase and suppression of phosphorylation and degradation of IκBα [74].

### 3.14 Gambogic Acid

Gambogic acid (GA), a xanthone derived from the resin of traditional Chinese medicine *Garcinia hanburyi* (mangosteen), blocks the STAT3 pathway, leading to suppression of growth and sensitization of cancer cells. Suppression of STAT3 phosphorylation by GA occurs through the inhibition of activation of the protein tyrosine kinases JAK1 and JAK2. Authors also found out that GA induced the expression of the tyrosine phosphatase SHP-1. Genetic silencing of SHP-1 by siRNA suppressed the ability of GA to inhibit STAT3 activation and to induce apoptosis, suggesting the critical role of SHP-1 in its action [75]. GA was also found to have a relevant effect on growth inhibition and apoptosis induction in MM cells, this activity being associated with the accumulation of ROS, which contributes to the activation of caspase-3 and the cleavage of poly (ADP-ribose) polymerase (PARP) [76]. Moreover, authors showed that GA suppresses hypoxia-activated pathways which are linked to MM progression, at least partly by the inhibition of the PI3K/Akt/mTOR pathway. In MM, the hypoxic BMM is an important factor driving tumor angiogenesis, which strongly correlated to disease progression and unfavorable outcome by activating the transcription factor hypoxia-inducible factor-1α (HIF-1α). The treatment with GA markedly decreased HIF-1α and VEGF expression under hypoxic conditions [77].

GA also suppressed osteoclasteogenesis *via* downregulation of CXCR4 and SDF-1α-induced chemotaxis of MM cells and downstream signaling of CXCR4. GA abrogated RANKL- and MM-induced differentiation of macrophages to osteoclasts in dose- and time-dependent manners [78].

# 4 Antibiotics

## *4.1 Mithramycin A*

Mithramycin A (MMA) is a DNA-binding, anti-tumor and neuroprotective antibiotic originally isolated from *S. grieseus* and previously used as a chemotherapeutic agent [79]. MMA interacts with the DNA minor groove at regions with high GC content in a non-intercalative manner, thus interfering with the expression of genes with GC-rich regions in their promoters (i.e. *DNMT1, SP1, etc.*) by preventing the binding of regulatory proteins [80].

MMA induces cellular differentiation and has been used to treat chronic myeloid leukemia and testicular cancer [81]. Moreover, it triggers anti-angiogenetic effects by inhibiting the expression of *SP1*-regulated genes, through the interaction with GC-rich sequences in DNA corresponding to *SP1* binding sites [82]. However, its cancer preventive potential has not been investigated yet. In MM patients, pioneristic studies prompted the use of MMA to manage with hypercalcemia and to control MM-related bone disease [83, 84]. Indeed, it was shown that sub-tumoricidal doses of MMA inhibit the release of calcium mediated by osteoclasts, and likely reduce their bone-resorbing activity [85]. More recently, MMA treatment resulted in a significant anti-MM activity against the syngenic 5TGM1 model. In this study, several endpoints of the disease were affected, such as BM invasion, malignant cell proliferation, cell cycle progression and angiogenesis [86]. Interestingly, the authors demonstrated that both anti-MM and anti-angiogenic effects were not mediated by the inhibition of SP1 but rather of c-Myc and by the activation of p53 and several anti-angiogenetic factors [86]. Of note, novel SP1 inhibitors, such as Terameprocol, a methylated derivative of nordihydroguaiaretic acid, have demonstrated significant anti-tumor activity in MM [87] and Waldestrom Macroglobulinemia [88], but their use as chemopreventive agents in hematologic malignancies has not been yet investigated.

## *4.2 Broad Spectrum Antibiotics*

Increasing studies support the idea of a functional link between inflammation and MM pathogenesis [7, 89]. Indeed, several microorganisms can activate both toll-like receptors (TLR) on MM cells as well as the production of proinflammatory cytokines (i.e. IL-6, TNF-α or IL-1) by the BM *milieu*, which then contribute to MM growth, survival and progression [87, 90, 91]. Consistently, there is evidence that many MM patients have severe bacterial infections just before diagnosis or immediately before disease progression [92] and different authors reported that a personal history of infection increases the risk of MM [93–95]. Moreover, clarithromycin has been shown to retain efficacy in association with corticosteroids and thalidomide and some evidence exists supporting its immunomodulatory and

anti-tumour effects in MM [96]. Altogether, these data prompted Valkovic et al. to propose the use of broad spectrum antibiotics as anti-MM prophylactic and/or therapeutic agents [92]. Specifically, the authors postulate that prophylactic treatments with antibiotics could suppress the pro-inflammatory *milieu* produced during recurrent bacterial infections, which represents one the most important factors leading to tumour growth and survival in MM patients. Even though this hypothesis appears extremely fascinating, future investigations are needed to clarify its real effectiveness.

## 5 Pharmacological Agents

### *5.1 Non-steroidal Anti-inflammatory Drugs (NSAIDs)*

Non-steroidal anti-inflammatory drugs (NSAIDs) are structurally different compounds which inhibit cyclooxigenase (COX) enzyme [97, 98]. These agents are mainly used for treatment of rheumatic diseases, but also relieve signs and symptoms of different types of inflammation [97, 98]. Over the past decade, pre-clinical studies demonstrated that NSAIDs could oppose to tumorigenesis through pro-apoptotic, anti-metastatic and immune-modulating mechanisms [99], and several epidemiological reports indeed suggested that the use of these compounds could be advantageous for prevention of several neoplastic diseases, including hematological malignancies [99]. In MM, we and others have already demonstrated a direct cytotoxic effect of different NSAIDs against patient MM cells and cell lines, both *in vitro* and *in vivo* [100–106].

The rational for the prophylactic use of NSAIDs basically relies on the established role of chronic inflammation in promoting MM pathogenesis [7, 89]. So far, major efforts have focused on aspirin (acetylsalicylic acid, ASA), the most popular NSAID, which inhibits COX-1 and COX-2 at a similar extent by covalently binding to the active site of these enzymes [97, 98]. A causal relationship between aspirin use and MM onset has been examined to date in five studies: one hospital-based case-control study (117 cases, 483 matched controls) [107], one population-based case–control study (179 cases, 691 frequency-matched controls) [93], and 3 prospective studies, the Vitamins and Lifestyle (VITAL) cohort (6–8 years follow-up, 66 cases of plasma cell disorders, cohort N = 64,839) [108], the American Cancer Society Cancer Prevention Study (CPS)-II Nutrition cohort (15 years follow-up, 310 cases, cohort N = 184,188) [109] and the Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS) (24 years follow-up for women, 12–18 years follow-up for men, 328 cases, cohort N = 16,381) [110]. Importantly, there is no evidence of any case-control study reporting an association between aspirin use with MM. Consistently, in the CPS-II study, neither quantity nor duration of aspirin use was associated with MM onset. Moreover, the VITAL study reported a significant inverse association

of regular use of 81 mg aspirin with risk of plasma cell disorders, but no evidence of association of plasma cell disorder risk with use of regular strength aspirin could be demonstrated. Conversely, the NHS-HP follow-up study reported an inverse association between regular use of aspirin (both quantity and duration) and the risk to develop MM. Overall, controversial results emerge from different studies, thus supporting the need of additional investigations.

## *5.2 DNMT Inhibitors*

Epigenetic changes are promising targets for cancer chemoprevention, since they occur early during carcinogenesis [111]. Indeed, hypermethylation of CpG islands generally anticipates the neoplastic onset and increases from pre-malignant lesions to invasive cancer [112–114].

DNA methyltransferases (DNMTs), the enzymes responsible for the methylation of DNA [115], are frequently upregulated in human cancer thus representing attractive targets for both therapeutic and chemopreventive interventions [116, 117]. DNMT-1 activity is pharmacologically inhibited by the cytidine analogs 5-azacitidine (Aza-C), 5-aza-2′-deoxycytidine (Aza-dC or decitabine) and zebularine, in which the carbon atom at position 5 in the pyrimidine ring has been replaced by a nitrogen atom [115]. Although these compounds were originally intended as cytotoxic drugs, it was next discovered that at low doses they could cause DNA demethylation by inactivation of DNMT-1 [115]. 5-Azacytidine (Aza-C), the prototype of DNMT inhibitors, was firstly developed and tested as a nucleoside antimetabolite in acute myelogenous leukemia [115]. Because of its relevant toxicity, other nucleoside analogs were preferred as therapeutics, such as Aza-dC and zebularine [118]. Differently from Aza-C, Aza-dC and zebularine are incorporated into DNA but do not interfere with RNA metabolism and protein synthesis [115]. Moreover, they also retain a lower mutagenic potential [115]. Importantly, it was demonstrated that both Aza-dC and zebularine effectively antagonize tumorigenesis in various genetic- or carcinogen-induced animal models of cancer [80]. Both compounds were tested in APC Min/+ mouse, which represents a widely established model for colon cancer prevention studies [80]. Strikingly, both Aza-dC and zebularine reduced by more than 95 % the number of adenomas when the treatment started at the age of 7 days, whereas Aza-dC was ineffective when delayed by 50 days [80, 119]. These data clearly point to early epigenetic events targeted by these compounds.

Several pre-clinical studies demonstrated a tumor-suppressive effect of DNMT inhibitors *in vitro* in MM [120–124]. This was mainly ascribed to re-activation of epigenetically-silenced genes, such as *SOCS-1, CDKN1A, CDKN2A, p53, PU.1* [120–124]. However, reports on their chemopreventive potential in MM patients are lacking.

### 5.3 HDAC Inhibitors

HDAC inhibitors (HDACi) retain structural similarities to the HDAC acetyl-lysine substrate, thus interfering with the function of these enzymes [33]. The majority of HDACi act as pan-inhibitors without selectivity to specific HDAC isoforms (i.e. Vorinostat and Trichostatin A) [33]. Selective HDACi, which affect either a single HDAC isoform (isoform-selective HDACi) or several isoforms within a single class (class-selective HDACi), indeed represent a new frontier of research [33]. Presently, several HDACi are under investigation within clinical trials, with vorinostat (Zolinza; Merck) and romidepsin (Istodax; Celgene) already approved by FDA for the treatment of advanced cutaneous T-cell lymphoma (CTCL) [125].

HDACi have also been investigated as chemo-preventive agents. Specifically, it was reported that vorinostat effectively delays the onset of lung cancer in A/J mice induced by either the tobacco carcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol or by vinyl carbamate [126, 127]. However, in these reports vorinostat was administered at doses near the maximum tolerated (500-600 mg/kg diet). Nonetheless, in an additional study, A/J mice were fed with vorinostat at only 250 mg/kg diet, beginning 1 week before injection with vinyl carbamate, for a total of 26 weeks [128]. Of note, also this report provided evidence of a prophylactic effect of this drug.

In MM, the benefit of HDACi as chemopreventive agents is still an unmet issue. However, different HDACi have already been tested in pre-clinical models of disease, showing promising anti-MM activity [125]. A common result from the majority of these studies has been the synergistic effect of HDACi when combined with novel or conventional anti-MM drugs, providing the rationale for combinatorial approaches [125]. Consistently, early-phase clinical trials have revealed only moderate activity of HDACi when administered as single agents in patients with advanced MM, but encouraging clinical response rates have been reported when combined with proteasome inhibitors, lenalidomide and dexamethasone [125].

Phenylhexyl isothiocyanate (PHI), a synthetic isothiocyanate, is an inhibitor of HDAC which also possesses hypomethylating activity in myeloma cells. Hypermethylation of *p15* and *p16* promoter CpG islands has been reported in MM clinical specimens and MM cell lines. The methylation status of *p15* and *p16* genes were not significantly different between MM and MGUS, nor in pre-treated and post-treated patients with MM. It was further demonstrated in MM patients that *p16* hypermethylation is associated with high plasma cell proliferation, higher β2-microglobulin concentration, and shorter survival, while no evident correlation was found with *p15* CpG island hypermethylation. Lu and colleagues found that PHI has dual epigenetic effects causing histone hyperacetylation and *p16* hypomethylation in RPMI-8226 MM cell line [129, 130].

## 6 microRNAs

microRNAs (miRNAs) belong to the most abundant class of small RNAs in animals representing approximately 1 % of the genome of different species. Each miRNA has hundreds of different targets: it has been estimated that approximately 30 % of the genes are regulated by at least one miRNA [131]. miRNA biogenesis occurs into the nucleus, where a pri-miRNA hairpin is transcribed by a RNA polymerase II and is subsequently cleaved by Drosha, a member of the RNA polymerase III family, in a 70–100 bp pre-miRNA that translocates in the cytoplasm; here, Dicer cleaves pre-miRNAs leading to 20–22 bp miRNA/miRNA* duplexes. After cleavage, the miRNA duplex is unwound by an as yet unknown RNA helicase and the mature miRNA strand binds to an Argonaute protein into a RNP complex that drives the mature miRNA strand to the 3′-UTR mRNA target sequence. 3′-UTR binding represses translation or induces mRNA decay, depending on the degree of complementarity between miRNA and its target [19, 132].

Noteworthy, by controlling the expression of target genes, miRNAs regulate pivotal biological functions such as cell proliferation, differentiation and apoptosis [133]. On this light, it is not surprising that dysregulation of miRNA expression is associated to perturbations of relevant molecular pathways leading to carcinogenesis [134]. miRNAs can act as either oncogenes (oncomiRs) or tumor suppressor genes, depending on their targets: oncomiRs are generally overexpressed in cancers and target tumor suppressor genes, while tumor suppressor miRNAs are frequently downregulated and inhibit the expression of oncogenic targets [19]. Interestingly, high expression of oncomiRs and low expression of tumor suppressor miRNAs have been detected in pre-malignant conditions [135], indicating that miRNAs may play a role in carcinogenesis. During the last 5 years, available information about miRNA expression in MM has rapidly grown [136–138], highlighting several miRNAs controlling critical genes in MM and revealing that miRNA expression pattern in MM is associated with specific genetic abnormalities. During the last 5 years, available information about miRNA expression in MM has rapidly grown [136–138], highlighting several miRNAs controlling critical genes in MM and revealing that miRNA expression pattern in MM is associated with specific genetic abnormalities.

In a pioneristic study, Pichiorri et al. analyzed miRNA expression profile in a panel of 49 MM cell lines, 16 bone marrow $CD138^{+}$ plasma cells isolated from MM and 6 from MGUS patients [139]. In this work, the authors found a common miRNA signature likely associated to the multistep transformation process of MM. Of note, they found miR-21, miR-106b-25 cluster, miR-181a and miR-181b upregulated in MGUS patients; moreover, by comparing MGUS and MM samples with normal plasma cells, they found some miRNAs, including miR- 32 and miR-17-92 cluster, upregulated only in MM cells [139]. Research performed by our group [140] indeed confirmed abnormal expression of miRNAs, with miR-29b, miR-125b, miR-199a-5p, miR-34a found expressed at low levels in MM cells [141–146], while miR-21, miR-125a-5p, miR-221, miR-222 upregulated in MM cells and acting as oncomiRNAs [90, 147–150].

As above discussed, dietary factors could exert a significant effect on the risk of cancer development and progression. Increasing evidence has demonstrated that modulation of miRNAs expression represents one of the key mechanism in the anticancer activity of a number of phytochemicals. For instance, in colon cancer cells resveratrol reduced the expression of oncogenic miRNAs, such as miR-17, miR-21, miR-25, while restored expression of downregulated miR-663 [151]. EGCG was found to induce apoptosis in in hepatocellular carcinoma by upregulating miR-16 thus resulting in reduced levels of its antiapoptotic target BCL-2 [152]. Furthermore, curcumin antitumor effects have been ascribed to upregulation of miR-15 and miR-16 in breast cancer cells [153], miR-203 in bladder cancer [154], and inhibition of miR-21 in colon cancer [155]. For an extensive report on miRNAs modulated by phytochemicals in solid tumors we recommend more specialized reviews [134]. Of note, literature regarding the involvement of miRNAs in phytochemicals' anti-MM action is scarce. Hu and colleagues found that berberine, a natural isoquinoline alkaloid extracted from many medicinal herbs, such as *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian), inhibited MM cell proliferation *in vitro* by down-regulating miR-21 expression [156], thus indicating that inhibition of this oncogenic miRNA is relevant for the anti-tumor activity of this compound.

Given that the chemopreventive effects of several natural or synthetic compounds has been ascribed to their capability to regulate the epigenome of cancer cells, miRNAs targeting DNMTs or HDACs could represent novel tools for chemoprevention.

In MM cells, miR-29b was found to target both DNMT3A and DNMT3B thus reducing global DNA methylation in MM cells and leading to apoptosis [117, 157]. Importantly, miR-29b was demonstrated to reactivate silenced tumor suppressor genes, such as *SOCS-1*, by inducing promoter-demethylation [158]. Moreover, miR-29b synthetic mimics potentiated 5-azacytidine-mediated inhibition of MM cell survival, this finding providing the preclinical framework for possible evaluation of miR-29b oligonucleotides/demethylating agents combination regimens in MM patients. The relevance of this family of miRNAs, named epi-miRNAs for their ability to target effectors of the epigenetic machinery, as well as their capability to control the epigenetic landscape by counterbalancing the epigenetic aberrations carried by cancer cells, is progressively emerging [157], and their potential role as chemopreventive agents indeed deserves in-depth investigation in the future.

## 7 Conclusions

There is now a wealth of encouraging data supporting the chemoprotective effects of natural compounds in experimental models of myelomagenesis. Notably, the anticarcinogenic activity of most of phytochemicals has been ascribed to their capability to restrain pro-survival signal transduction pathways, mainly STAT3, NF-κB and AKT, thus exerting potent cell growth inhibitory and apoptosis inducing effects not only *in vitro* in MM cell lines and primary samples, but also *in vivo* in human MM preclinical mouse models. Moreover, certain natural compounds may inhibit the expression and/or the activity of epigenetic enzymes, such as DNMTs or

HDACs, which have been found deeply deregulated in MM plasmacells, thus exerting a wider activity on MM cells [28, 29].

However, detailed information on specific targeting and biovailability of phytochemicals, along with large scale clinical trials in MM patients, are needed in order to develop anti-MM commercial drugs to be used for preventing or therapeutic purposes.

Along with natural compounds, endogenous non-coding RNAs are emerging as epigenetic regulators endowed with anti-MM activity. Of note, abnormal miRNA expression correlates with MM-specific cytogenetic abnormalities and is now regarded as an intrinsic feature of MM growth and progression. Early studies have also established that miRNA signatures are endowed with prognostic power and could predict outcome and treatment in MM [19]. A subclass of miRNAs, named epi-miRNAs, acts by downregulating epigenetic enzymes, thus reverting aberrant epigenetic patterns found in MM cells and representing novel candidates for epigenomic approaches in cancer prevention or therapy [157, 159]. Importantly, many phytochemicals have been demonstrated to alter miRNA levels by epigenetic mechanisms, this finding adding another layer of complexity in phytochemicals' mechanism of action [134]. However, similarly to phytochemicals, the translation of miRNAs into clinics requires the development of safe delivery vehicles as well as more in-depth analyses on their pharmacokinetic and pharmacodynamic properties.

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# Targeting MicroRNAs: Molecular Basis of Cancer Prevention

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## 1 Introduction

Carcinogenesis is a process in which normal cells become cancerous cells. Carcinogenesis consists of multiple steps and multiple disorders of cellular signaling pathways. In normal cells, many genes regulate cellular signal transductions and guide cells to divide (proliferation), to die (apoptotic cell death), to develop (differentiation), or to repair themselves when damaged by environmental carcinogen. The genes, which control cell proliferation, apoptotic cell death, differentiation, and repairing, malfunction because of mutations and other molecular defects during the onset of carcinogenesis. In addition to gene mutations, epigenetic alterations also significantly contribute to carcinogenesis which is mediated through the regulation of gene expression due to DNA methylation or histone modification even though there is no alteration on DNA sequences. During the processes of carcinogenesis, the aberrations in the cellular signaling caused by gene mutations or epigenetic alterations lead to over-proliferation and less apoptotic cell death. The cells with DNA damage or epigenetic aberrations do not die as they should for maintaining the normal homeostasis while newly dividing cells with DNA damage or epigenetic aberrations result in tumor formation. It is well accepted that

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carcinogenesis consists of three serial stages including initiation, promotion, and progression. The strategies for cancer prevention could target these three stages to block the initiation or suppress and reverse the promotion and progression of cancer. To that end, cancer chemopreventive agents act at all three different stages through regulation in the expression of critical genes and thereby altering cellular signaling, leading to the control of cell proliferation, differentiation, senescence, or apoptosis [1]. Recently, dietary or natural agents have received increasing attention in cancer prevention because these agents could prevent, suppress, and reverse carcinogenic progression through the regulation of microRNAs (miRNAs)-gene nexus signaling.

The miRNAs are a group of naturally occurring, evolutionarily conserved, noncoding small (20–22 nucleotide long) RNA molecules which negatively regulate gene expression by suppressing translation or decreasing mRNA stability [2, 3]. The discovery of miRNAs has significantly improved our knowledge on the regulation of genes. Emerging evidences have demonstrated that miRNAs play important roles in the regulation of gene expression in many physiological conditions. The biosynthesis of miRNAs includes several linked steps. The miRNA genes are first transcribed by RNA polymerase II to form pri-miRNAs which have comparative longer sequences. Then, under the mediation and digestion by Drosha and Dicer, pri-miRNAs are shortened to form pre-miRNA duplex and move from nucleus to cytosol. After duplex dissociation, the mature single stranded miRNAs bind to the 3′UTR regions of target mRNAs with the RNA-induced silencing complex (RISC), resulting in target mRNA silencing through translational repression or mRNA degradation [4–6]. Through the regulation of target gene expression, miRNAs are known to control cell proliferation, differentiation, and apoptosis [7–9]. Therefore, impaired miRNA expression has been implicated in carcinogenesis [6, 7]. Recent research has found that certain miRNAs function as tumor suppressors which suppress oncogene expression while several miRNAs are oncogenic modulator inhibiting cancer suppressors. Higher expression of oncogenic miRNAs and lower level of cancer suppressive miRNAs have been found in pre-cancerous cells [10], suggesting the aberrant miRNAs as one of many molecular basis of carcinogenesis. Interestingly, recent studies have shown that several natural and dietary agents could normalize the deregulated expression of miRNAs, suggesting that these agents could serve as promising cancer preventive agents mediated through regulation in the expression of miRNAs.

It is well known that dietary factors could exert a significant effect on the risk of cancer development and progression. Growing evidence has demonstrated that a healthy diet, physical exercise, and maintenance of healthy body weight are very important in lowering the risk of cancer. Some components in the healthy diet provide compounds that have anti-carcinogenic activity, as well as the activity that inhibits the proliferation of cancer cells. Recently, several natural and dietary agents which show no systemic toxicity have been considered as nutraceuticals, and thus they have been investigated for their effects on the inhibition of carcinogenesis and suppression of tumor growth which is in part mediated through the regulation of miRNAs [11–15]. It has been found that these natural agents

(for example, isoflavone, curcumin, resveratrol, 3,3′-diindolylmethane, vitamins, etc.) could regulate the levels of miRNA expression, resulting in the suppression of carcinogenesis and inhibition of tumor progression. However, some dietary and environmental factors such as smoking or over-nutrition could also regulate the expression of miRNAs that leads to the development of cancers, suggesting that one must deregulate the cancer promoting miRNAs and also regulate the miRNAs that would be beneficial for the prevention of carcinogenesis, prevention of tumor progression and also for cancer therapy. In the following section, we will succinctly summarize the state of our knowledge on the role and regulation of miRNAs in different cancer system and the summary of agents that could serve to deregulate miRNAs toward cancer prevention and therapy.

## 2 The miRNAs Related to the Exposure to Environmental Factors and Gene Instability During Carcinogenesis

During the processes of carcinogenesis, genetic and epigenetic factors as well as environmental factors all are important because they collectively contribute to the molecular changes in cells. The gene instability and the exposure of environmental factors such as infection, ultraviolet light, irradiation, and environmental toxicants could alter expression levels of miRNAs, resulting in the altered expression of genes and cellular signal transductions (Fig. 1). The aberrant expression of miRNAs and genes with disorder of cellular signaling transductions initiates the development of cancers and also promotes the progression of cancers. In the following section, we will succinctly summarize what is known in regards to the deregulation of miRNAs under different conditions of gene-environment interaction.

### *2.1 Genetic Susceptibility*

Personal genetic susceptibility because of single nucleotide polymorphisms (SNPs) and mutation of genes including miRNA genes plays important role in carcinogenesis. It has been found that about 10 % of human pre-miRNAs possess SNPs. The SNPs could lead to the alterations in the expression levels of miRNAs, and thus affects the expression levels of their target mRNAs, which in part, initiates the processes of carcinogenesis [16]. Moreover, the SNPs in the mature miRNA seed region or target binding site have been found to be correlated with cancer risk, suggesting their critical roles in carcinogenesis [17–20]. Studies have shown that the SNPs in miR-196a were significantly associated with increased risk of cancers including lung, breast, esophageal, hepatic and gastric cancers [17–21]. The SNPs in the miR-499 together with cigarette consumption has been shown to increase the risk of squamous cell carcinoma in the oral cavity [22]. In addition, SNPs at k-Ras genes in complementary site of let-7 has been found to be significantly linked with increased risk for non-small cell

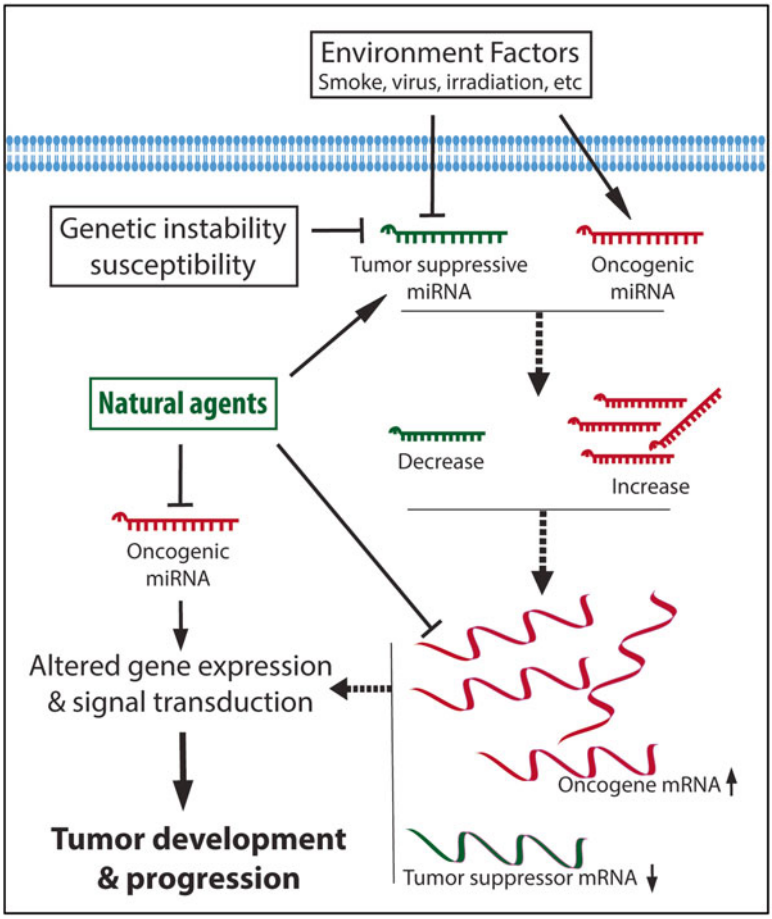


**Fig. 1** The role of miRNAs in carcinogenesis and cancer progression, and the effects of natural agents on the expression of miRNAs

lung cancer in smokers [23]. It has also been found that metastatic colorectal cancer patients with the polymorphism at k-Ras let-7 LCS6 had poor drug response in the clinical setting [24]. These limited findings provide examples, suggesting the important role of miRNA gene instability in carcinogenesis.

## *2.2 Smoking*

Studies have found that smoking-induced lung carcinogenesis involves the alterations in the expression of miRNAs [25–28]. These extensive literature in the deregulated expression of miRNAs are clear examples that these miRNAs are associated

with cigarette smoke and progressive development of lung cancers. Cigarette smoke causes lower expression of several miRNAs such as let-7 which could alter stress response, apoptosis, proliferation, and angiogenesis [27]. Moreover, the loss of expression of miRNAs such as let-7a could become irreversible upon constant exposure to cigarette smoke [25]. The irreversible silencing of specific miRNAs is usually caused by miRNA gene deletions, which are frequently observed in smoking-induced lung cancer [29]. Therefore, the re-expressing let-7 by chemopreventive agents or administrating pre-let-7 itself in early stages of lung cancer could be a promising strategy for the prevention of smoking-induced lung cancer.

Indeed, in a study investigating the preventive effects of let-7 in lung carcinogenesis, let-7a was introduced into bronchial epithelial H727 cells which were exposed to cigarette smoke [30]. The cigarette smoke down-regulated let-7a, leading to the anti-apoptosis in the cells. Twenty percent of H727 cells survived at high doses of cigarette smoke. However, when let-7a was introduced in H727 cells, the survival of H727 cells exposed to high doses of cigarette smoke was decreased to 3 %. These results demonstrate that let-7a is anti-carcinogenetic factor in lung and that administrating let-7a could be a promising strategy to prevent lung carcinogenesis. In addition to administration of let-7 itself, animal study showed that chemopreventive agents could protect the down-regulation of miRNAs including let-7 caused by cigarette smoke [31–33]. In addition to let-7, other miRNAs such as miR-218 and miR-21 could also be deregulated by cigarette smoke, leading to the deregulation of oncogenes or tumor suppressors [32–34]. These reports collectively suggest that cigarette smoking leads to the deregulation in the expression of critical miRNAs which, in part, defines the molecular basis of lung carcinogenesis.

## *2.3 Radiation*

It has been well accepted that ultraviolet light is tightly associated with the onset of melanoma. Epidemiologic studies have found that intense intermittent ultraviolet light exposure and severe sunburns during childhood confer the highest risk of melanoma [35]. Studies have shown that ultraviolet light radiation increased the levels of miR-203, miR-205, miR-21, and miR-22 which inhibits the expression of PTEN in skin cells, and perhaps may target the expression of many other genes, suggesting the role of these miRNAs in skin carcinogenesis [36, 37]. It has also been found that miR-376c, miR-23b, miR-452, and miR-132 were significantly up-regulated while miR-487b, miR-494, miR-miR-10a, and miR-210 were significantly decreased in human keratinocytes after 6 h of ultraviolet light irradiation [38]. In addition, a study on genomic mutations of melanoma have shown that melanoma has intensively decreased binding of miRNAs to 3′UTRs of mRNA because of the mutated gene mRNA [39].

In our environment, we are exposed to low dose of radiation every day. Radiation could be carcinogenetic; however, radiation is also used for cancer therapy. The miRNA could be altered in response to radiation-induced DNA damage and genomic

instability which are the factors that contribute to carcinogenesis. In addition, some miRNAs such as miR-24 could suppress H2AX, leading to the reduced sensitivity of cells to gamma-irradiation [40, 41]. Growing investigations have shown that miRNAs expression profiles of various cancer cells could be altered upon radiation. The investigations are also focused on the miRNA expression patterns which are correlated with the sensitivity of cancer cells to radiation therapy. It has been found that up-regulation of miR-126, let-7a, miR-495, miR-451 and miR-128b could increase the sensitivity of lung cancer cells to radiation-induced apoptosis [42]. Other miRNA expression patterns also play important roles in the sensitivity of cancer cells to radiation therapy [5]. This knowledge could be harnessed for designing miRNA-targeted approach for maximizing the cancer killing effects of radiation for cancer therapy. On the other hand limiting environmental exposure to radiation will minimize the deregulated expression of miRNAs, and thus prevent carcinogenesis.

### *2.4 Other Factors*

In addition to smoking and radiation, inflammations caused by viral infection are also responsible for carcinogenesis. It has been found that hepatitis C virus could increase the expression of miR-155 in hepatic cells, resulting in hepatic cancer growth and progression [43]. Human papillomavirus and its oncogene could also regulate the expression of several miRNAs including miR-34a, let-7, and miR-155, suggesting that these miRNAs may function as the key modulators of carcinogenesis in virus-related cancers [44–48]. In addition, the Epstein-Barr virus is highly linked with nasopharyngeal carcinoma. Epstein-Barr virus could suppress the expression and function of miR-203 through the oncoprotein latent membrane protein 1, leading to the carcinogenesis of nasopharyngeal carcinoma [49]. These results collectively suggest that alterations in the expression of miRNAs are linked with many environmental factors that contribute to carcinogenesis, and thus deregulation in the expression of many of these miRNAs by molecular approaches or through dietary modifications could be useful for the prevention and/or the inhibition of tumor progression for human cancers.

## 3 The Altered miRNA Expression Profiles of Different Human Cancers

The miRNAs could be oncogenic or tumor suppressive dependent on their functions in the regulation of genes during carcinogenesis. Growing investigations have been focused on the profiles of miRNA expression in different cancers. It is known that all types of solid and hematopoietic tumors possess aberrant expression of miRNAs. It is common knowledge that the oncogenic miRNAs are up-regulated while the cancer suppressive miRNAs are down-regulated in cancerous cells. The deregulated

miRNAs cause alterations in the expression of genes and cellular signal transduction, leading to the development and progression of cancers. In the following section, we will summarize what is known in some selected human solid tumors.

## 3.1 Lung Cancer

Lung cancer is the leading cause of cancer death in men and women in the United States. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. The aberrant expressions of miRNAs have been found in NSCLC. Many studies focusing on the identification of miRNA biomarkers in the development and progression of NSCLC have revealed that several miRNAs exhibit significantly differential expression in NSCLC tissue compared to normal lung tissues. Among them, miR-22 and miR-448 function as oncogenes whereas miR-654-3p and miR-181 function as tumor suppressors to inhibit the progression of NSCLC [50, 51]. Moreover, a study on the expression levels of miRNAs in plasma of early stage of NSCLC has been conducted to assess the diagnostic miRNA biomarkers for NSCLC [52]. It has been found that the levels of five miRNAs including miR-20a, miR-223, miR-21, miR-221 and miR-145 in plasma of NSCLC patients were significantly deregulated compared with the healthy subjects. Among them, plasma miR-20a, miR-223, miR-21 and miR-145 are better predictive markers for smokers whereas miR-155 is more suitable for non-smokers [52]. Studies were also focused on the miRNA profiles in the tissues and serum of early stage NSCLC. It has been found that the expression of miR-29c in both tissues and serum was significantly increased and that miR-93 expression was also up-regulated in NSCLC tissues whereas the level of serum miR-429 was down-regulated in NSCLC [53]. In addition, miRNA expression profiles have been used to identify progressive potential of lung adenocarcinoma, and it is found that miR-411, miR-370, and miR-376a were significantly associated with cell migration, cell adhesion, and poor survival [54]. These results suggest the value of miRNAs as biomarkers in lung cancer, and it is our understanding that this information could be useful for the development of miRNA-based therapeutics in the near future.

## 3.2 Colon Cancer

Colorectal cancer is another common cancer in men and women in the United States. The aberrant expression profiles of miRNAs have also been exploited for diagnosis and prognosis in colorectal cancer. The differentially expressed miRNAs including miR-21, miR-103, miR-93, miR-31 and miR-566 have been observed in colorectal cancer [55]. Importantly, the up-regulation of miR-21, miR-93, and miR-103 together with down-regulation of miR-566 predict the metastasis and lymph node invasion of colorectal cancer [55]. The studies are also focused on the role of

serum miRNAs in colorectal cancer. It has been found that several miRNAs (including miR-320a, miR-629, miR-720, miR-877. miR-484, miR-500, miR-194, miR-210, and miR-378) were up-regulated whereas several miRNAs (including miR-151, let-7d, miR-103, miR-107, miR-652, miR352, miR-409, miR-146a, miR-221, miR-199a, and miR-744) were down-regulated in serum from stage IV patients with metastasis, suggesting that serum miRNA expression profile could be useful for prediction of metastatic colon cancer [56]. In addition, studies have also shown that miR-362, miR-218, and miR-320 could function as tumor suppressors to induce cancer cell apoptotic death, inhibit cancer cell proliferation, and suppress the invasion of colorectal cancers [57–60]. Collectively, this knowledge must be exploited for developing miRNA-based therapeutics.

### *3.3 Prostate Cancer*

Prostate cancer is the second leading cause of cancer related death among men in the United States. Studies on the miRNA expression profiles showed that the expressions of miR-125b, miR-21, miR-126, miR-151a, miR-221, miR-20a, miR-148a, miR-200b, miR-375, and miR-222 were significantly increased while the levels of miR-143, miR-145, and miR-486 were significantly decreased in prostate cancer cells, suggesting the role of aberrant expression of miRNAs in the development and progression of prostate cancer [61–63]. Further study showed that miR-548c was up-regulated in prostate cancer stem cells and in castration-resistant prostate cancer [64] and that enforced overexpression of miR-548c in differentiated cells induced stem-like properties and radioresistance [64], suggesting that miR-548c could promote the self-renewal of cancer stem cells and the progression of prostate cancer. Moreover, miR-34 is a cancer suppressor miRNA, and deceased levels of this miRNA have been found in prostate cancer. Because of the methylation in the promoter of miR-34 gene, the level of miR-34a is very low [65], which leads to the down-regulation of its target androgen receptor (AR) and Notch1 gene expression. Similarly, tumor suppressor miR-29a has been found to be down-regulated in prostate cancer cells due to miR-29a gene methylation, which leads to higher expression of its target gene TRIM68, and thus may cause in the progression of prostate cancer [15]. These results suggest that non-toxic demethylating agents must be developed for the re-expression of selected miRNAs toward prostate cancer therapy, and to that end nutraceutical may play important role.

### *3.4 Breast Cancers*

Breast cancer is the most common cancer in women in the United States. Early diagnosis is an important strategy for lowering mortality. It has been found that miR-484 is significantly up-regulated in serum from patients with early breast

cancer compared to healthy volunteers, suggesting the value of miR-484 in the detection of early breast cancer [66]. The aberrant expressions of miRNAs are also observed in breast cancer cells [67–69]. The miRNA expression profile of human breast cancer showed that the expressions of miR-21 and miR-200c were altered and that the levels of their target gene PDCD4 and PDCD10 were accordingly altered, leading to the occurrence of breast cancer [70]. Triple negative breast cancer is the most aggressive breast cancer. It has been found that the aberrant expressions of several miRNAs are linked with triple negative breast cancer. The expression of let-7a was significantly down-regulated in triple negative breast cancer compared to normal breast tissues [71]. Moreover, the level of miR-200c expression was also significantly decreased in triple negative breast cancer with BRCA mutation. The low expression of miR-200c was associated with higher expression of VEGFα, suggesting that low expression of miR-200c is responsible for angiogenesis and progression of triple negative breast cancer [71]. In addition, the expressions of miR-573 and miR-578 were down-regulated in breast cancer with BRCA mutation and was found to be associated with angiogenesis [72]. Studies have also shown that miR-183/miR-96/miR-182 cluster is highly expressed in most breast cancers, resulting in rapid mitosis and also promoted cell migration and survival [73]. These results demonstrate the critical role of aberrant miRNAs in the formation and progression of breast cancer, and thus these and other miRNAs could the potential therapeutic target.

### *3.5 Pancreatic Cancer*

Pancreatic cancer is a highly aggressive malignancy, and the fourth leading cause of cancer related death in the US. The expression profiles of miRNAs in pancreatic adenocarcinoma have been investigated [74–77]. It has been found that the expressions of miR-21, miR-155, miR-210, miR-221, and miR-222 were up-regulated in pancreatic cancer compared to normal pancreatic tissue and that the levels of miR-31, miR-122, miR-145, and miR-146a were decreased in pancreatic cancer [77]. Further analysis showed that miR-21 and miR-155 were tumorigenic and that the levels of miR-21 and miR-155 were linked with tumor stage and poor prognosis [77]. Studies also found that miR-1247 was significantly decreased in pancreatic cancer tissues and that high expression of miR-1247 expression was associated with higher overall and recurrence free survival in pancreatic cancer patients, suggesting the value of miR-1247 for prognosis [78]. In addition, miR-150 has been found to be a tumor suppressor in pancreatic cancer. More excitingly, a nanoparticle-based miR-150 delivery system has been designed and tested *in vitro*. Treatment of pancreatic cancer cells with nanoparticle-based miR-150 showed efficient intracellular delivery of miR-150 mimics which significantly reduced the expression of MUC4, one of the targets of miR-150 [79]. More importantly, nanoparticle-based miR-150 was found to inhibit cell proliferation, clonogenicity, motility, and invasion of pancreatic cancer

[79], suggesting the therapeutic value of nanoparticle-based miR-150. Therefore agents like this and other potential agents could be the future of medicine for the treatment of human pancreatic cancer.

### 3.6 Hepatic Cancer

Hepatocellular carcinoma is one of the most highly malignant cancers in the United States, and its incidence is rapidly increasing. The aberrant expressions of miRNAs have been observed in hepatocellular carcinoma. It has been found that the expressions of miR-22, miR-221, miR-222, miR-29a, miR-320b, miR-320c, miR-31, miR-372, miR-93, and miR-96 were significantly up-regulated while the levels of miR-29c, miR-373, miR-520b, and miR-520e were decreased in hepatocellular carcinoma [80, 81]. In addition, miRNA expression profiles of hepatitis C virus-associated hepatocellular carcinoma have also been investigated. The expressions of miR-122, miR-100, and miR-10a were up-regulated while the expression of miR-198 and miR-145 were significantly down-regulated in hepatitis C virus-associated hepatocellular carcinoma [82]. The mechanistic study showed that the up-regulated miR-222 expression was common in hepatocellular carcinoma, leading to the metastasis of hepatocellular carcinoma through the activation of AKT signaling [83]. Another mechanistic experiment demonstrated that the level of miR-124 was commonly lower in hepatocellular carcinoma and that the lower expression of miR-124 was significantly correlated with aggressive behavior and poor prognostic feature of hepatocellular carcinoma [84]. The low expression of miR-124 led to induced expression of its target ROCK2 and EZH2, and subsequently promoted invasion and metastasis of hepatocellular carcinoma, suggesting the role of miR-124 in hepatocellular carcinoma suppression. AS stated above for other human solid tumors, the existing knowledge on hepatocellular carcinoma associated miRNAs could become the targets of future therapeutics.

### 3.7 Gastric Cancer

Epstein-Barr virus (EBV) is one of the major oncogenic viruses and highly linked with nasopharyngeal carcinoma. However, it was also found in nearly 10 % of gastric cancer. In EBV-associated gastric carcinoma, several miRNAs including ebv-miR-BART7-3p, ebv-miR-BART4-5p, and ebv-miR-BART1-3p were found to be highly expressed [85]. The up-regulation of ebv-miR-BART4-5p could inhibit Bid expression and apoptosis in EBV-associated gastric cancer, suggesting the carcinogenetic role of these ebv-miRNAs [85].

Lower expressions of miR-148a and miR-34 are commonly observed in gastric cancers. The hypermethylation in the promoter of miR-148a gene has been observed in gastric cancer cells [86]. In addition, the low expression of miR-148a has been

correlated with tumor size and invasion in gastric cancer [86]. These results suggest that miR-148a is a tumor suppressor and contributes to the inhibition of cancer progression in gastric cancer. Another tumor suppressive miRNA, miR-10b, also low expressed in gastric cancer because of epigenetic regulation [87]. Re-expression of miR-10b in gastric cancer cells down-regulated its target Tiam1 and suppressed cancer cell growth, migration, and invasion [87], demonstrating the cancer suppressive function of miR-10b in gastric cancer. The above mentioned reports suggest that novel non-toxic demethylating agents could be promising for the treatment of gastric cancer.

### *3.8 Multiple Myeloma*

Multiple myeloma is a hematological malignancy in which abnormal plasma cells aberrantly proliferate and accumulate in the bone marrow. It has been found that miR-29b was significantly down-regulated in primary malignant plasma cells and multiple myeloma cell lines [88]. Enforced expression of miR-29b into multiple myeloma cells inhibited cell proliferation and promoted apoptosis through the inhibition of its targets, MCL1 and CDK6 [88, 89]. In addition, MCL1 is also a target of miR-137 and miR-197. In multiple myeloma cell lines and multiple myeloma patient samples, the expression level of miR-137 and miR-197 was found to be significantly lower compared to normal plasma cells. Enforced expression of miR-137 and miR-197 into multiple myeloma cells also significantly reduced MCL1 protein expression, leading to the induction of apoptosis and the inhibition of cell proliferation, colony formation and migration of multiple myeloma cells [90]. Similarly, miR-30-5p which functions as a tumor suppressor, and the expression level of miR-30-5p has found to be lower in multiple myeloma [91]. The low expression of miR-30-5p increases the expression of its target BCL9 which is a transcriptional coactivator of Wnt signaling, leading to myeloma cell proliferation, survival, migration, drug resistance, and the formation of multiple myeloma cancer stem cells [91]. In contrast, the high expressions of miR-181a, miR-20a and miR-451 were observed in multiple myeloma, and the inhibition of these miRNAs enhanced the effectiveness of anti-myeloma drug bortezomib [92, 93]. All these findings suggest that targeting miRNAs could be a novel and efficient therapeutic approach for multiple myeloma [94–96].

### *3.9 Other Tumors*

Other solid and hematopoietic tumors also showed aberrant expression of miRNAs. The miRNA expression profiles of oral squamous cell carcinoma, esophageal cancer, thyroid carcinoma, large B-cell lymphoma, cutaneous T-cell lymphoma, cutaneous large B-cell lymphoma, Epstein-Barr virus-associated NK/T-cell lymphoma,

acute lymphoblastic leukemia, B cell non-Hodgkin lymphoma, Hodgkin lymphoma, and chronic lymphocytic and acute lymphocytic leukemia have been investigated [97–107]. The miRNA expression profiles of these tumors may provide newer molecular basis for the prevention and/or treatment of these tumors using miRNA-targeted therapeutic approaches.

## 4 Dietary or Natural Agents Regulate the Expression of miRNAs

Because altered expressions of miRNAs are significantly related to the formation and progression of various cancers, targeting those aberrantly expressed miRNAs may be a novel approach for the prevention and/or treatment of cancers. Recently, growing studies have focused on the investigation of dietary or natural agents that could be used for the regulation of miRNAs and thereby subsequent suppression of oncogenesis and cancer progression. Growing evidences have shown that dietary or natural agents such as isoflavone, curcumin, resveratrol, 3,3′-diindolylmethane, vitamins, etc. have anticancer properties, at least in part, through the regulation of miRNAs (Fig. 1). These dietary and natural agents could regulate cellular signal transduction through the modulation of miRNAs, resulting in the suppression of oncogenesis and cancer progression, thus miRNA-targeted therapeutic strategies could be useful for the prevention and/or treatment of cancers [108, 109]. In the following section, we will summarize the state-of-our-knowledge on miRNA-targeting effects of selected natural agents.

### *4.1 Regulation of miRNAs by Isoflavone*

Isoflavones are rich in soybean and have been shown to have anti-carcinogenesis activity [110, 111]. Studies have shown that isoflavone could modulate the expression of miRNAs and normalize the levels of several miRNAs in precancerous and cancerous cells. The mechanisms underlying the miRNA regulation by isoflavone includes epigenetic regulation (such as DNA methylation and histone modification) [112, 113]. The effect of isoflavone on the expression of miRNAs in prostate cancer cells has been found which was as similar as the effect of demethylating agent 5-aza-2-deoxycytidine, suggesting that the modulation of miRNAs by isoflavone occurs, in part, through epigenetic regulation [114]. Isoflavone caused significant inhibition of miR-125a, miR-125b, miR-15b, and miR-320 expression, and a significant induction in the expression of miR-548b-5p in prostate cancer [114]. It has also been found that the promoters of miR-29a and miR-1256 were hypermethylated in prostate cancer cells, leading to the lower expression of miR-29a and miR-1256 in prostate cancer cells [15]. More importantly, isoflavone decreased the

methylation of miR-29a and miR-1256 promoters and thereby induced the expression of miR-29a and miR-1256 [15]. This study also showed that isoflavone up-regulated the level of TRIM68 and PGK-1, which are the targets of miR-29a and miR-1256. The miR-1260b is an oncogenic miRNA in prostate cancer tissues. Isoflavone genistein treatment significantly decreased the level of miR-1260b in prostate cancers through epigenetic regulation, resulting in the inhibition of proliferation, invasion, and migration of prostate cancer cells [115]. In addition, isoflavone genistein also epigenetically modulated the expression level of miR-145, miR-221, and miR-222, resulting in inhibition of cancer cell proliferation [116, 117].

Isoflavones genistein could also inhibit the expression of miR-27a and thereby suppress cancer cell growth and invasion in various cancers [118, 119]. Isoflavone also increased the expression of miR-146a and thereby inhibited invasion of pancreatic cancer cells through the down-regulation of EGFR, NF-κB, IRAK-1, and MTA-2 [120]. Isoflavone could also induce the expression of miR-200 in gemcitabine-resistant pancreatic cancer cells, leading to the down-regulation of EMT markers including ZEB1, slug, and vimentin, and the sensitization of pancreatic cancer cells to gemcitabine [121]. In UL-3A and UL-313 ovarian cancer cells, isoflavone genistein regulated a total of 53 miRNAs, resulting in the inhibition of cancer cell proliferation [122]. In prostate cancer cells, genistein down-regulated the expression of miR-221 and miR-222, leading to the expression of tumor suppressor gene ARHI and thereby causing inhibition of cancer cell growth and invasion [116]. All these results demonstrate the effect of isoflavone on the modulation of miRNAs, and the inhibition of carcinogenesis and cancer progression which clearly suggest that it is possible to further develop isoflavone-based cancer preventive and/or therapeutic strategies through targeting miRNAs.

### *4.2 Regulation of miRNAs by Curcumin*

Curcumin is a major constituent of turmeric [123, 124]. It has been well known that curcumin has anti-inflammation, anti-oxidant, and anti-cancer effects [124–126]. Recent studies showed that curcumin could modulate the levels of miRNA expression, in part, through epigenetic regulation. The miR-203 is a cancer suppressive miRNA. In bladder cancer, the level of miR-203 was significantly down-regulated, primarily because of hypermethylation in promoter regions of miR-203 gene [127]. Importantly, curcumin induced the expression of miR-203 in bladder cancer via demethylation of the methylated miR-203 promoter. The increased miR-203 expression decreased the expression levels of Akt2 and Src, which are miR-203 target genes, resulting in the suppression of bladder cancer growth [127]. In addition, curcumin modulated the expression of miRNAs through the control of the enzymes in the epigenetic regulation [128]. Curcumin has been shown to regulate the expression of histone deacetylase (HDAC), histone acetyltransferases (HATs), DNA methyltransferase I (DNMT1), and miRNAs, leading to the inhibition of cancer cell

growth [129]. Curcumin could also up-regulate the expression level of miR-29b and down-regulate the level of DNA methyltransferase 3b (DNMT3b) expression, resulting in increased PTEN expression which leads to the inhibited cell growth and the increased apoptotic cell death [130].

In breast cancer, curcumin increased the level of miR-22, leading to the down-regulation of estrogen receptor and transcription factor Sp1, causing the inhibition of invasion and metastasis of cancer [131]. Curcumin also up-regulated the expression of miR-15a and miR-15b, leading to the down-regulation of Bcl-2 expression in breast cancer [132]. In lung cancer, curcumin decreased the expression of miR-186 and, in turn, increased the level of its target caspase-10 expression, leading to the induction of apoptotic cell death of lung cancer cells [133]. In colon cancer, curcumin down-regulated miR-21 and subsequently up-regulated PDCD4, leading to the inhibition of cancer cell proliferation, invasion, and metastasis [134]. Although these pre-clinical studies are exciting as to the effects of curcumin in the deregulation of specific miRNAs, the activity of curcumin *in vivo* is very limited due to the complexity of its low bioavailability.

To overcome the lower bioavailability of curcumin *in vivo*, curcumin analog CDF was designed and synthesized by our group. It was shown that CDF could regulate the expression of miRNAs through epigenetic regulation. In colon cancer, the expression of miR-34 was reduced partly due to the methylation in the promoter of miR-34 gene; however, CDF or 5-aza-2-deoxycytidine increased the levels of miR-34a and miR34c, reduced the expression of miR-34a target gene Notch 1, and suppressed the growth of colon cancer [135], further suggesting that the effects of CDF on miRNAs was in part mediated through epigenetic regulation. Moreover, CDF also increased the level of miR-101 and, in turn, inhibited the expression of EZH2, one of the targets of miR-101. Furthermore, CDF could induce the expression of let-7, miR-26a, miR-146a, and miR-200, leading to the inhibition of cell growth, sphere forming, and invasion of pancreatic cancer cells [136]. These findings demonstrate that curcumin analog CDF could modulate miRNA-mRNA nexus, and thus CDF could become a new agent for the prevention and/or treatment of human malignancies.

### *4.3 Regulation of miRNAs by Resveratrol*

Resveratrol can be found in human diets such as grapes, mulberries, and peanuts [137], and has been shown to possess anti-oxidant and anti-carcinogenesis activity [138–140]. Resveratrol has shown its inhibitory effect on carcinogenesis through regulation of miRNAs. It has been known that resveratrol significantly increased the levels of miR-129, miR-204, and miR-489 through the suppression of DNA methyltransferases (DNMT1 and DNMT3b) [141]. Resveratrol also modulated the epigenetic signal transduction in the miR-520h/PP2A/Akt/NF-κB/

FOXC2 signaling cascade, resulting in the suppression of growth of lung cancer cells [142]. In prostate and gastric cancers, resveratrol inhibited the expression of oncogenic miRNAs including miR-17, miR-21, miR-22, and miR-106, and thereby led to the induction of PDCD4, and other targets [143–145]. In colon cancer, resveratrol increased the expression of tumor suppressor miR-663, and thereby, down-regulated TFG-β signaling [146]. In benzo[a]pyrene-7,8-diol-9,10-epoxide-transformed human bronchial epithelial cell, resveratrol treatment increased the levels of another tumor suppressor miRNA, miR-622, which led to the inhibition of k-ras and tumor growth *in vitro* and *in vivo* [147]. In lung cancer, resveratrol significantly down-regulated the expression of miR-92a and up-regulated the expressions of miR-194, miR-299, miR-338, miR-582, and miR-758, which led to the inhibition of lung cancer cell growth [148]. These results clearly suggest that resveratrol could serve as a miRNA-targeted chemopreventive and/or therapeutic agent.

### *4.4 Regulation of miRNAs by Indol-3-carbinol (I3C) and 3,3′-Diindolylmethane (DIM)*

I3C is a phytochemical mainly derived from cruciferous vegetables [149, 150]. DIM is the *in vivo* self-dimerized product of I3C. I3C and DIM have been shown to have anti-carcinogenesis and anti-cancer activities [151–153]. In lung cancer, I3C inhibited the expression of oncogenic miRNAs including miR-21, miR-31, miR-130a, miR-146b, and miR-377, leading to the up-regulation of tumor suppressor PTEN, PDCD4, and RECK [154]. In gemcitabine-resistant pancreatic cancer, DIM has been shown to induce the expression of miR-200 family, let-7 family, and miR-146a, leading to the reversal of EMT, inhibition of invasion, and induction of sensitivity to gemcitabine through the regulation of MET, EGFR, and NF-κB signaling [120, 121]. In prostate cancer, androgen receptor (AR) signaling has been known to contribute to the development and progression of prostate cancer and also the development of castrate resistant prostate cancer [155]. It was shown that the silencing of miR-34a could activate AR signaling in prostate cancers. An experimental study from our group showed that DIM or 5-aza-2-deoxycytidine could demethylate the methylation in promoter of miR-34a in prostate cancer cells, suggesting that epigenetic regulation contributes to the decreased miR-34a and that DIM could increase its level of expression through demethylation regulation [65]. It was concluded that the up-regulation of miR-34a by DIM could suppress AR and, thereby, PSA in prostate cancer cells. Moreover, in the study on prostate cancer tissues, we found that DIM intervention before radical prostatectomy up-regulated miR-34a and down-regulated its target genes, AR, PSA and Notch-1, in tissue specimens [65], suggesting that up-regulation of miR-34a by DIM intervention could effectively inhibit AR signaling which indeed is useful for prostate cancer therapy.

### 4.5 Regulation of miRNAs by Vitamins and Other Nutrients

Vitamin A is an important nutrient for humans and it is critical for cell growth and differentiation. The miRNA expression profile altered by vitamin A in acute promyelocytic leukemia cells has been investigated. It was found that the expressions of miR-186, miR-215, and miR-223 were up-regulated whereas the expressions of miR-17, miR-25, miR-193, miR-195, and let-7a were down-regulated [156], suggesting the differential effects of vitamin A on cells. However, another similar study showed somewhat different results. Furthermore, vitamin A treatment down-regulated the expression of miR-181b and up-regulated the expressions of miR-15a, miR-15b, miR-16-1, let-7a, let-7c, let-7d, miR-223, miR-342, and miR-107 [157], suggesting the anti-proliferative effects of vitamin A.

Similar to vitamin A, folic acid which is a type of B vitamin has been studied. An animal study showed that a diet deficient in folic acid could induce hepatocellular carcinoma in rat which was in part mediated through the down-regulation of miR-122 [158]. Folic acid could also decrease the expression of miR-10a and block ethanol-induced teratogenesis in fetal mouse [159]. Moreover, low folic acid intake could cause high expression of miR-222 in lymphoblastoid cells and that folic acid supplementation has been shown to normalize the level of miR-222 [160].

Vitamin D is a well-studied chemopreventive agent, which could modulate miRNAs in various cancer cells in support of its biological activity. Vitamin D3 has been shown to reduce the expression of miR-181, leading to the up-regulation of p27 and subsequent cell cycle arrest of human myeloid leukemia cells [161]. Vitamin D also induced the expression of miR-22, leading to the inhibition of cell proliferation and invasion of colon cancer cells [162]. It is known that vitamin D binds to its receptor and exerts its chemopreventive effect, which is in part supported by the results showing that miR-125b and miR-22 regulate the level of vitamin D receptor; therefore, these miRNAs participate in the process of chemoprevention by vitamin D [162, 163].

Epidemiologic studies have shown the chemopreventive effect of vitamin E in various cancers. An experimental study showed that vitamin E deficiency in rats could down-regulate the expression of miR-122a and miR-125b, which regulates lipid metabolism and tumorigenesis, suggesting that vitamin E could protect hepatocyte from carcinogenesis through the regulation of miRNAs [164]. Another dietary agent, selenium has been considered as chemopreventive agent. In prostate cancer, selenium induced the expression of miR-34, leading to the transcriptional activation of p53 and the induction of apoptosis [165], which could be the mechanism by which selenium inhibits carcinogenesis. Although the above mentioned studies are limited with respect to their effects on miRNAs, these information could however be useful for designing appropriate clinical studies in the future to ascertain the beneficial effects of vitamins for cancer prevention and therapy through targeting miRNAs.

## 5 Conclusions and Perspectives

In conclusion, emerging evidences have shown that the alterations in the expression of miRNAs are very common in human malignancies, and it is our perspectives that specific miRNAs may play critical roles in the processes of carcinogenesis and cancer progression. During the process of carcinogenesis, genetic and epigenetic factors together with environmental factors, typically known as gene-environment interaction, contribute to the alterations in the molecules that are involved in gene regulation and consequently cellular signal transductions inside the cells. The gene instability and the exposure of environmental factors such as infection, ultraviolet light, irradiation, and environmental toxicants could regulate the level of expression of miRNAs, leading to the alterations in gene expression and cellular signal transductions. The aberrant expression of miRNAs and genes with disorder of cellular signaling transductions initiates carcinogenesis and subsequently promotes the progression of cancers. The aberrant expression of miRNAs is being appreciated as the molecular basis of cancer development and progression. The altered miRNA expression profiles have been found to be significantly associated with tumor diagnosis, grades, and prognosis. Therefore, targeting the altered miRNAs could be a promising strategy for cancer prevention and treatment. It is known that dietary factors could influence the processes of carcinogenesis; interestingly, dietary or natural agents (collectively could be called as nutraceuticals) have been found to regulate and normalize the expression of miRNAs. The non-toxic nature of dietary and natural agents such as isoflavone, curcumin, resveratrol, I3C, DIM, vitamins, etc. exert their beneficial effects on cancer prevention and treatment. To that end, the *in vitro* and *in vivo* animal and human intervention studies have shown that these agents could up-regulate cancer suppressive miRNAs and down-regulate oncogenic miRNAs, and thereby may lead to the suppression of cancer formation and progression in humans. Hence, these agents could prevent the occurrence and/or recurrence of cancers. Moreover, treatment with these non-toxic agents for regulating the expression of miRNAs together with cytotoxic chemotherapeutics could become a promising strategy for future cancer therapy.

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# The Liquid Biopsies: A New Important Step in Cancer Research

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## 1 The Tumor Disease and Its Diagnosis: Flying Below the Radar

The last decades have witnessed great advances in the use of body imaging [1], protein biomarker analysis [2] and molecular profiling [3–5] for cancer diagnosis. The advances of imaging techniques and molecular profiling have helped both to detect the disease in early stages as well as to refine the histological classification of cancer, which have led to more specific treatments with less side effects and increased effectiveness [6]. It also has brought us closer to the goal of personalized medicine, with the arrival of biomarker-guided strategies that individualize the management of the disease in all the phases of care [7].

However, as more we get close to personalized medicine (individual, precise diagnosis and targeted therapies) it is becoming clear that the traditional diagnosis and detection techniques are not enough to provide the accuracy, sensitivity and specificity that is needed to answer for the new challenges. Amongst these challenges we can enumerate:

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### 1.1 Diagnosis of Tumor Beyond the Sensitivity Threshold of Current Methods

The diagnosis of many cancer diseases frequently uses levels of blood protein biomarkers that display low sensitivity and specificity [8]. Several of the protein biomarkers used for diagnosis nowadays, like the carcinoembryonic antigen (CEA) for colorectal cancer, CA-125 for ovarian cancer, CA 19-9 for pancreatic cancer and PSA for prostate cancer, have issues regarding a low sensitivity and specificity, particularly in the context of early disease [9]. An example is the report of the U.S. *Preventive Services Task Force* (*USPSTF*) discouraging the use of PSA screening for prostate cancer detection [10]. Moreover, some types of cancer diseases are not detectable until advanced stages are reached [11, 12]. As a consequence, patients receive a poor prognosis at the time of diagnosis, which left them with scarce therapeutic approaches available [12]. A paradigmatic case is the non-small cell lung cancer (NSCLC). This disease is normally diagnosed at Stages III-IV [13, 14], leading to a poor prognosis, and it is one of the reasons behind its high mortality rate [12]. Even in cancer types where the early diagnosis have been a breakthrough, like breast cancer, the spread of tumor cells through the blood/lymph circulation from the primary tumor to distant sites also escapes the detection by standard imaging or biomarker methods [11, 15].

### 1.2 Analysis of the Heterogeneous Composition of the Tumor

Into the light of the last discoveries of genomic, biomolecular and cell biology research, tumors have been defined as cell conglomerates with a polyclonal profile [16, 17]. This profile changes in response to external inputs, like the treatment and the influence of the tumor microenvironment (immune system activity, metabolism, reactive species of oxygen (ROS levels), $O_2$ levels…). This environmental pressure makes the best adapted cells to survive, which in turn makes the disease evolve into a new profile since the last scan or biopsy, and therefore, can result in a non-effective or even harmful therapy choice. Moreover, if we approach the disease in early stage, it becomes necessary to obtain a more detailed profile of the tumor heterogeneity in order to select the appropriate therapy as soon as possible.

### 1.3 Continuous Follow-Up

As stated previously, tumor heterogeneity involves swift changes in tumor response to treatments due to selection of clones resistant to the treatment, changes in the tumor microenvironment and the appearance of *de novo* mutations. These changes have been linked to the progression and relapse of the disease [18]. The need of

follow-up is mandatory for targeted therapies, since its high specificity can be hampered as soon as the tumor acquires resistance mechanism [18, 19]. Tissues biopsies provide important information, but are not useful for continuous follow up as the invasiveness of the procedure limits its availability. This situation is more crucial in metastatic diseases, where it is regularly impossible to obtain biopsies from the different distant sites.

## 2 The Liquid Biopsy. Concept and Potential

The new discoveries in genomics, biotechnology, biochemistry and molecular pathology have unraveled a large number of potential tumor biomarkers for clinical use [20]. Together with the discovery of new sources of biomarkers in the body fluids of cancer patients, a new concept has arisen: the liquid biopsy [21–23]. This concept was first used to define the detection of circulating tumor cells (CTCs), and their potential as biomarkers of disease progression and outcome [22]. However, over the last decade the concept has expanded, nesting many other components and thus, becoming one of the most promising source of information for early diagnosis, follow-up of disease progression and the appearance of resistance to treatments. The liquid biopsy nowadays is defined as *a biomarker that can be isolated from any body fluids* (*blood*, *urine*, *saliva*…) *and that represents the tissue from which it originates* [7]. By definition, the liquid biopsy presents some advantages over the other techniques of diagnosis. As it analyzes components in body fluids [24–26], is not as invasive or stressful as tumor biopsies or imaging techniques. This turns out in less complication on the procedure, and also may be more affordable since tumor biopsies and imaging techniques require expensive equipment and expertise to be performed accurately.

If analyzed with new techniques of next generation sequencing (NGS) like microarray technology or proteomics, the liquid biopsies reveal themselves as sources of many potential biomarkers. Moreover, as these new techniques make possible to detect biomolecules in very low quantities, detection of the disease in early stages becomes feasible beyond the sensitivity threshold of conventional techniques. The liquid biopsies may also allow the acquisition of a complete profile of the tumor heterogeneity and obtain comprehensive knowledge about new features of the molecular profiling of the disease. The non-invasiveness of the sample extraction allows for a continuous follow-up of the disease progression, as opposed to the snapshot of the state of the disease that are tumor biopsies [20]. This kind of follow-up will make it easier to detect the swifts into the tumor behavior and genetic payload, which can be critical into the appearance of resistance mechanisms, and in consequence, on the treatment selection.

The variety of components of the liquid biopsy will allow, as the research on these topic progresses, to obtain a wider picture of the state of the disease, allowing the clinicians to tap into the communications between the cancer cells and the tumor environment (immune cells, metastatic niches, distant metastatic sites, extracellular

matrix and even between the different clones in the tumor). This entire new source of information has the potential to monitor the reaction of the tumor to the treatments and its environment, allowing for more rational based targeted therapy, and will even be able to reveal new therapeutic targets.

The last years of research in the field of liquid biopsy has identified components that have been highlighted as a source of biomarkers for early stage detection. So far, the best known components are the CTCs, the circulating tumor DNA (ctDNA), circulating tumor microRNA (ctmiRNA) and the extracellular vesicles (microvesicles and exosomes). These components have revealed a new level of complexity into cancer disease, as all of them have proven to be an active agent into disease development [27–30].

## 3 CTCs: The Pioneers of Liquid Biopsy

Discovered in the nineteenth century, CTCs were the first component of liquid biopsies to be analyzed. CTCs are detached cells from the primary tumor that migrate to the lymphatic and blood system [31]. They are present in low quantities, even in advanced disease status (cut-off values are in the range of 5–7.5 CTCs/7.5 ml of blood for several cancer types). Once they have detached, they face different fates: they can invade distant tissues [31, 32], clutch together and get stuck in blood vessels [15] or they became dormant cells, reactivated later on as metastatic distant sites [33, 34]. Amongst the factors that condition its fate is the transition to a epithelial-mesenchymal (EMT) phenotype, which is identified as a factor for CTCs to start micrometastatic sites [15, 31].

The detection of CTCs has been traditionally related to advanced disease and metastasis appearance [23, 32, 35, 36]. However, there is growing evidence that highlights the CTCs as predictive and prognostic biomarkers in early stages, as their shedding from the tumor mass can occur even in the first stages of the disease [37]. The CTCs have been reported, amongst others, as biomarkers in patients of breast [36, 38–40], prostate [27, 41, 42], colorectal [43–46] and lung cancer [35, 47–49]. Is not surprising then that CTCs have been incorporated for its use in clinic as biomarkers. In the year 2004, the FDA approved the CellSearch® system as the first method to detect CTCs for monitoring cancer progression [50], based in a clinical trial on metastatic breast cancer, where CTCs presence and cut-off value were linked to prognosis and monitoring of the disease progression [50, 51]. Other clinical trials in different cancer types have highlighted as well its potential as predictive biomarkers. For example, in prostate cancer, several clinical trials have linked the oscillation in the level of CTCs with the treatment response, confirming the role of CTCs as a surrogate biomarker [52–55]. Another approach for CTCs as biomarkers, is the analysis of the molecular alterations that they carry, as they could be a reflection of the alterations present in the tumor mass [15, 56]. As an example, it has been reported that the EGFR mutation T790M, indicative of resistance to tyrosine kinase inhibitors (TKI), can be detected with high specificity in CTCs of NSCLC patients.

Moreover, its detection can be related to reduced progression-free survival (PFS) [52]. Also, ALK translocation could be detected through Fluorescent In-Situ Hybridization (FISH) and immunohistochemistry (IHC) of CTCs of NSCLC patients [57]. However, their utility as markers for prevention and early detection of the disease is uncertain, because of their genetic plasticity and the fact that CTCs can undergo a transition to EMT phenotype that both make them no longer represent the tumor mass phenotype, and also escape detection methods based on epithelial antigens. When we consider their low numbers in early stage disease, it becomes clear that new techniques of detection are needed to make them suitable for clinical use [15]. Intensive research is in motion to validate different techniques of detection of CTCs in early stage of disease and link its concentration and molecular profile with disease progression and outcome [15, 31, 58]. Nevertheless, more sensitive techniques are needed to detect them to decide whether or not are valuable as biomarkers in the early stages of the disease [15, 43, 51, 58, 59].

As for the other components of liquid biopsies, the cell-free nucleic acids (ctDNA and ctmiRNA) and the exosomes are more promising as a source of biomarkers for early diagnosis and prediction. The rationale is that they are present in higher concentration in blood and other fluids of cancer patients [24, 60–62] in comparison with CTCs, which makes them, in principle, more suitable for isolation and analysis. Even if the cancer cells are present in low numbers, their high activity guarantees the production of these components at rates high enough to make them easier to be detected.

## 4 The ctDNA. Multiple Approaches for Cancer Monitoring

There is no consensus in the literature whether the fraction of DNA present in extracellular vesicles is taken up in the definition of the total extracellular DNA pool. Nevertheless, here we accept the definition of cell free DNA (cfDNA) by Peters et al. as *the fraction of extracellular occurring DNA* (*eoDNA*) *that is free of relation with any subcellular or molecular structure* [28]. Our target for biomarker purposes is the fraction of cfDNA that has its origin from the cancer cells. This extra cfDNA is normally referred as circulating tumor DNA (ctDNA). In normal physiologic circumstances, necrotic and apoptotic cell debris are removed from the tissue by infiltrated phagocytes. Combined with the accelerated cellular turnover, this process is not as effective in cancer cells, leading to an increase of necrotic and apoptotic cell debris [24, 63]. In consequence, the content of apoptotic and necrotic bodies that is released into the bloodstream, including ctDNA, is much higher than in normal conditions [64]. Despite this knowledge, the exact mechanisms of ctDNA biogenesis are yet not clear [65, 66]. Moreover, as ctDNA main source was thought to be DNA fragments released from dying cells of the primary tumor, metastatic or CTC cells [65], active mechanism of secretion of ctDNA from cancer cells has been proposed as well [66]. However, the regulation of this mechanism of secretion is still unclear [58, 65, 66].

The value of ctDNA as a biomarker has been analyzed in different ways. The absolute value of ctDNA was historically the first used biomarker, as the ctDNA is present in higher concentrations in the blood of cancer patients than in healthy individuals, although with great variation between serum and plasma in both groups [65, 67].

Another approach is to evaluate the integrity of ctDNA. In the same way that apoptosis and necrosis are altered in cancer cells, the cellular debris generated is also aberrant. In normal conditions, the fragments of DNA that are detectable in blood have their origin in apoptosis events, consistently generating small fragments of nucleosomal DNA ranking between 185 and 200 base pairs (bp) [68]. In cancer cells, however, both the increased necrotic events and the deregulation of apoptotic reactions generate a great variety of DNA fragments, with heterogeneous lengths, due to the incomplete and random digestion of DNA [69]. Consequently, DNA integrity in plasma (DIA), which is defined as the ratio between longer and the shortest fragments, could be used as biomarker to monitor cancer progression [70]. This approach has been tested in patients of breast [71] and colorectal [70] cancer.

However, the use of the DIA ratio has two major downsides. In one hand, as in the overall evaluation of ctDNA levels, there are benign processes, like inflammation or trauma, that concur with the cancer disease and produce high levels of cfDNA [65] that alters the DIA ratio, which can render useless the DIA as a biomarker in some cancer types, or in some stages of the disease progression. On the other hand, the manipulation of the blood sample for ctDNA is a critical parameter, since the inadequate manipulation induces the death of blood cells, which release fragments of DNA into the extracellular space, further altering the content and proportion of DNA fragments [58].

Another approach for the analysis of ctDNA integrity has also been tackled from the analysis of the ALU or LINE1 repetitions present in the ctDNA [71]. In this kind of analysis, tumor specificity is lost, but its increased sensitivity has the potential to become an universal blood biomarker for different cancer types [65].

With the arrival of NGS tools, such as digital droplet PCR analysis (ddPCR), ctDNA has regained attention as a biomarker, as we are able to sequence the fragments of circulating ctDNA [7, 64]. This analysis is based on the fact that the ctDNA carries tumor-related genetic and epigenetic alterations [65]. This approach solves the problem of the specificity of the ctDNA, as these alterations are characterized in cancer cells. Amongst the alterations analyzed, we can detect loss of heterozigosity or mutations in oncogenes or tumor suppressors, which are critical for the treatment with targeted therapies [61]. All these approaches have resulted in several reports of ctDNA analysis for molecular alterations in several cancer types [58, 61, 72, 73]. An example of the potential prognostic biomarker of molecular alteration analysis in ctDNA is the detection, before and after surgery, of PI3K mutation in early-stage breast cancer patients, with an specificity of 100 % and sensitivity of 93 % [73]. However, there is a gap between these discoveries and their application in clinical environment that need to be solved before incorporating the ctDNA analysis as diagnostic biomarkers into clinical practice [58, 67, 74].

## 5 cfmiRNA: New Regulation Twist in Cancer Disease, New Options for Diagnosis

The microRNAs, or miRNAs, are small (18–25 nucleotides), non-coding, endogenous, single-stranded RNAs [75]. They were discovered only a couple of decades ago [75, 76], and since then big efforts have been made to reveal their role as regulators of gene expression [77]. The number of known miRNAs has experienced an exponential increase in the last decade. Initiatives like the mirBase®, (v21, June 2014, www.mirBase.org), shows 2588 mature miRNAs entries in humans [8, 78]. Nevertheless, it has been recently reported that the number of human miRNAs is probably vastly superior [79].

The biogenesis of miRNAs begins with its transcription by RNA polymerase II [80] or III [81] into kilo-base-long primary miRNA transcripts, or pri-miRNA [82]. Next, the transcript is processed in the nucleus into pre-miRNA, roughly 70 nt long, by a nuclear microprocessor complex formed by RNAse III Drosha and DGCR8 [75, 83]. After being transported to the cytoplasm by the exportin-5/Ran GTPase complex, miRNAs undergo a maturation process in the cytosol [84]. This maturation is performed by the RNAse III Dicer, which generates a mature miRNA duplex of roughly 22 nt long. At this point, the mature, double-strand miRNA is bound to the RNA induced-silencing complex (RISC) [75, 85]. In the RISC complex, the passenger strand is degraded and the mature strand interacts effectively with RISC, which is the actual effector of the physiological miRNA effects [84–86]. All these processes offer many opportunities for the regulation of miRNA expression, with the recognition of miRNA-specific differences in the biogenesis, opening a plethora of regulatory options to express and process differently individual miRNA [84]. Moreover, many transcription factors, like p53 or c-Myc, and the changes of the methylation patterns in the miRNA gene promoter, have also been reported to regulate miRNA expression [82, 84, 86].

As for their biological relevance, microRNAs have been reported to be involved in crucial biological processes, such as development, tissue differentiation, apoptosis and cell proliferation [87]. These activities of miRNA emerge from the specific interaction between the miRNA and the RISC complex. The RISC complex is guided by the mature strand of miRNA to interact, through the Argonaut-2 (Ago2) proteins, with the 3′ non-translated region (3′-UTR) of specific messenger RNAs (mRNAs) [84, 86]. This interaction can result in target mRNA cleavage, repression of the translation or its deadenylation [84]. Another important feature of the function of miRNAs is that one single miRNA can modulate the expression profile of numerous sets of proteins [88, 89]. All together, these discoveries gave a robust rationale to analyze the miRNA profile in cancer [78, 90, 91]. The first link between miRNA alterations and cancer was found in 2002, with the discovery that the mir15-a/16-1 cluster in the 13q14 human chromosome is absent in chronic lymphocytic leukemia (CLL) [92]. Thus, it was deduced that this miRNA have a tumor suppressor effect, as it was further

reported that this miRNA downregulate the expression of the oncogene Bcl-2 [93]. Since then, there is a consensus that in the context of cancer, miRNAs have a role as oncomiRNAs or tumor suppressor miRNAs, in relation to the mRNA that interact with and how the expression of the miRNA is altered [8, 91]. Moreover, the discovery of stable miRNA in different body fluids [91, 94, 95] has also boosted the research of miRNA as a biomarker for the last decade [8, 78, 96], both in tissue and body fluids.

The advances of NGS and digital-droplet polymerase chain reaction (ddPCR) have approached the miRNA analysis to the clinical environment [8, 58] in several cancer types:

In NSCLC, serum levels of four miRNAs (miR-486, miR-30d, miR-1 and miR-499) have been significantly associated with OS [97]. In another report, nine circulating miRNAs (miR-221, miR-660, miR-486-5p, miR28-3p, miR-197, miR-106a, miR-451, miR-140-5p, and miR-16) were reported to be related with a risk of lung cancer malignancy and a poor prognosis [98]. This report also suggests that specific miRNA signatures in pre-disease plasma samples are able to predict and discriminate the development of the more aggressive, early metastatic tumors, which are frequently undetectable by yearly spiral-CT. Furthermore, a phase I/II biomarker study identified two circulating miRNA (mir-15b and mir-27b) that were capable of discrimination between healthy donors and NSCLC patients, proving their potential to screen for NSCLC in early stages [99]. In another report, high serum levels of miR-21 were significantly related to lymph node metastasis, advanced clinical staging, and a poor prognosis in NSCLC patients [100].

In lung squamous cell carcinoma (SCC), five miRNAs (miR-205, mir-19a, mir-19b, mir-30b, and mir-20a) have been reported to be significantly downregulated after tumor resection, making them potential biomarkers for screening of the disease [95].

In breast cancer, a pilot study reported that the miRNA profile in serum of breast cancer patients has several altered miRNA signatures in respect to healthy volunteers [101–103]. However, a branch of patients participating in the large, prospective Sister Study was recently evaluated, looking for useful miRNA signatures for early diagnosis, with inconclusive results [104]. Furthermore, five miRNA signatures (miR-21, -145, -34a, -10b and -221) that were previously reported to be related to breast cancer have recently been reported to show no correlation between its levels and clinical parameters [105].

Overall, there is growing evidence that miRNAs are useful as biomarker in many cancer types [13, 91, 106]. Several reviews recently published will help the reader to deepen further into this subject [8, 78, 107, 108].

However, it is also evident that there are some issues for the utility of miRNAs as a biomarker in liquid biopsies that hampers its arrival to the bedside. First, as it happens with cell-free DNA, measurement of circulating miRNA levels is challenging because of contamination by varying levels of cellular miRNAs present in the body fluids [58]. Secondly, the evidence that correlates the circulating miRNA levels with the levels of miRNAs in the tumor tissue, or with the disease

progression and outcome is not conclusive due to the different approaches of isolation and analysis, although it is well established that miRNA levels in tumor tissue are selectively altered compared with normal tissue [58, 104]. It is also necessary to discover endogenous or housekeeping miRNA, common to cancer disease, or into every type of cancer disease, in order to normalize miRNAs measurements between the different samples tested, thus facilitating the comparison between different studies [8, 58, 109] Finally, our knowledge of miRNAs as a regulatory agent in cancer disease is on its early stages, as we have only scratched the surface of the miRNAs that are involved in the different processes of the disease appearance and progression.

Another issue is that the whole picture of the circulating miRNAs and its role in cancer disease is not fully understood, since miRNA is present in the body fluids in different compartments, like lipoprotein and Ago2 complexes, that are responsible for its stability in body fluids [8, 110, 111]. Recently, it has been reported that one of the main sources of miRNAs in body fluids are the miRNAs contained in the exosomes [111–114].

## 6 Exosomes: The Messenger Pigeons of Cancer Disease

The cells in our bodies are in continuous communication with other cells, close or distantly, which is fundamental for any multicellular system to be sustainable. Amongst the different ways to communicate, cells exchange information through secretion [115]. At first, it was thought that the secretion of molecules between cells was restricted to small molecules, growth factors and chemokines [116]. In recent years, it was discovered that a family of microvesicles, including the exosomes, could have an important role in intercellular communication [117, 118].

The exosomes were first described in the 1980s, but the term was only branded several years afterwards [119]. At first, exosomes were considered degenerated fragments of dead cells [119–121], but by the end of the millennium some reports described exosomes secreted by immune cells [118, 122, 123]. As a new cellular compartment, exosomes harbor a selective payload of biomolecules that may have an important role in different cellular processes [60]. These discoveries have given the start to great efforts from the research community to understand the origin and function of the exosomes. Over the last few years a considerable amount of experimental evidence has demonstrated that the transmission of information through exosomes is a sophisticated method of cell communication [30, 116, 124–127]. Many cell types secrete their own set of exosomes, such as hematopoietic cells, epithelial cells, neural cells, adipocytes, fibroblasts, stem cells and many types of tumor cells [124, 125, 128]. The exosomes have been reported to be involved in many physiological and pathological conditions, including cancer [62, 121, 129–131].

## 6.1 Biogenesis of Exosomes

The biogenesis of exosomes starts at the endosome pathway, with its first steps being common to the recycling of membrane components into lysosomes, a well-known mechanism for elimination of transmembrane proteins or an excess of cell membrane. Distinction can be made at the level of the formation of intraluminal vesicles (ILV) inside of multivesicular bodies (MVB) [124, 128]. The outer membrane of the cell is incorporated into an early endosome through endocytosis, trafficking the proteins present in the outer membrane of the cell to the inner side of the early endosome [121, 124, 128]. Next, the now mature endosome undergoes a budding process of its membrane to form several nano-sized ILV per endosome. It is important to note that after this process, the membrane proteins in the ILV have the same orientation that they had in the cell membrane, which is important for the biological role of exosomes and its clinical utility [114, 132].

Afterwards, the MVB can be signaled to degradation in the lysosomes, or it can migrate to the outer part of the cell (Fig. 1), where it fuses with the cell membrane, causing the ILV to be released into the extracellular space. From that moment on, they are properly called exosomes [124, 128].

At the moment, the details of exosome biogenesis and its regulation are not fully understood [121, 124]. Two pathways of biogenesis are proposed: one is dependent of the endosomal sorting complex required for transport (ESCRT) [133]. In this proposed pathway a growing group of auxiliary proteins is involved [133–135] that assists the ESCRT complex, and also a widespread family of Rab-GTPases that provides the energy for the vesicle formation, protein trafficking and exocytosis of the vesicles [136]. This model is supported by the detection of both ESCRT and accessory proteins in the lumen of the exosomes [113, 121, 124]. The other biogenesis pathway has been described as ESCRT-independent, but dependent in sphingomyelinase activity and also related to ceramide biosynthesis [137]. As our knowledge of exosome biogenesis expands [114], it has been proposed that the different pathways could be simultaneously active and generate different exosome subpopulations [128, 138].

## 6.2 Exosome Molecular Composition and Biological Functions

Exosomes are conformed by a lipid bilayer similar to the one of the cell membrane, which allows a high degree of stability of their content, providing a safe environment to the different biomolecules that they contain [21]. Amongst them, we can find proteins and several types of nucleic acids.

Our knowledge of exosome protein content has grown exponentially over the last years, due to several initiatives, like ExoCarta, which attempts to compile the known payload of exosomes [139]. So far, 4563 proteins have been identified in

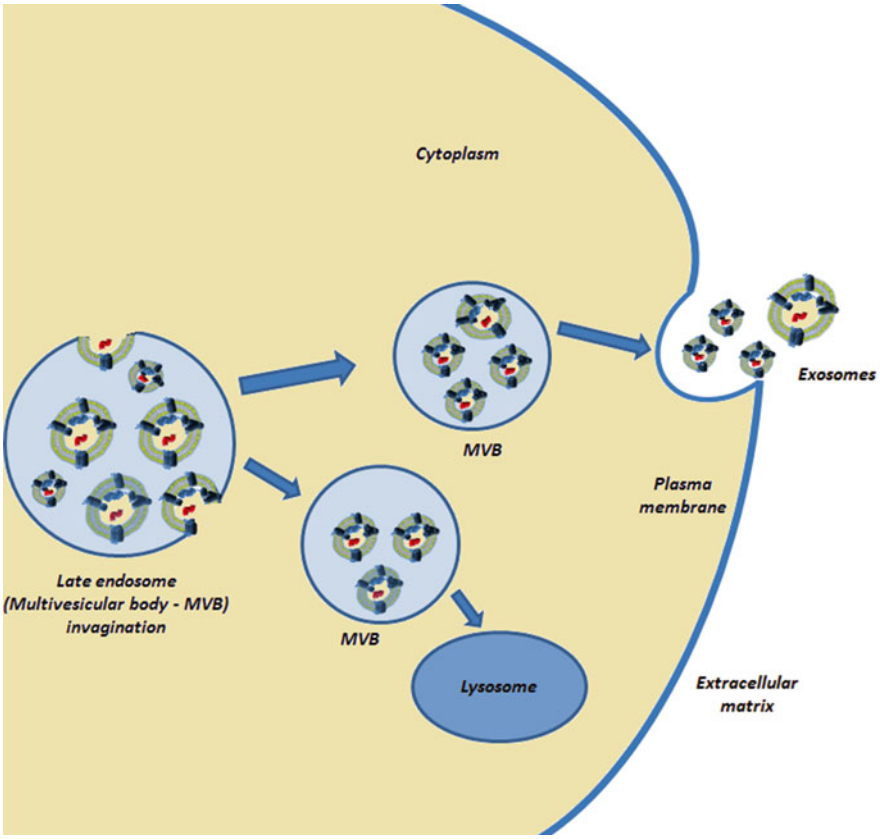


**Fig. 1** Exosome biogenesis

exosomes. The proteomic studies have helped to define a list of proteins which are common to exosomes. Amongst them, we can list heat-shock proteins (Hsp60, Hsp70, and Hsp90) membrane proteins, lipolytic enzymes, cytoskeletal proteins, GTPases, major histocompatibility complement I and II (MHC I, MHC II), tetraspanins (CD63 and CD81) and proteins involved in MVBs biogenesis (Alix and TSG101) [140]. Because these proteins have been consistently identified in exosomes, it was proposed that some of them, like CD63, CD81, Alix and TSG101, could be used as markers for exosome characterization [121, 124]. However, it is a point of discussion that these proteins are common to other microvesicles as well. Besides, exosomes from the ESCRT-independent pathway lack some of these markers. In this context, their role as markers for exosome isolation should be reassessed [121, 141, 142].

So far, lipidomic analysis has revealed the presence of 194 different lipids in exosomes. The lipid bilayer is enriched in raft-associated lipids, including sphingolipids, such as sphingomyelin and ceramide, cholesterol and diglycerides [143]. Several other lipids, like phospholipids, glycerophospholipids, eicosanoic acid and biologically active lipids like prostaglandins and leukotrienes, have been detected

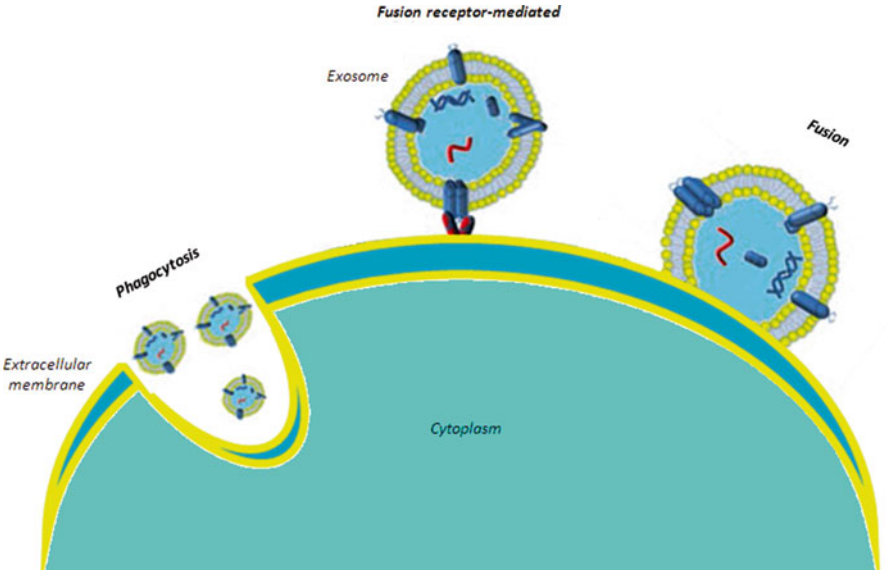


**Fig. 2** Different exosome uptakes by target cell

as well [117]. This specific lipid composition provides exosomes with a rigid structure that may explain their stability in biological fluids and cell culture mediums [111, 143].

The exosomal nucleic acids are a very complex milieu. They have been reported to carry significant amounts of messenger RNAs, microRNAs (miRNAs), mitochondrial DNA (mtDNA), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small-nuclear RNAs (snRNAs), small-nucleolar RNAs (snoRNAs) and long non-coding RNAs (lncRNAs) [144, 145]. MicroRNAs studies have discovered the presence of 764 different miRNAs in exosomes, subdivided in miRNAs with oncosuppressor functions, with oncogenic effects (usually called oncomiRNAs), related to apoptotic pathways or involved in angiogenesis, amongst others [112, 113, 146]. Multivesicular bodies have been linked to miRNA effector complexes, indicating the existence of specific mechanisms for a selective packaging of miRNAs into exosomes [113].

Exosome uptake (Fig. 2) by target cells can occur through receptor-ligand interaction, fusion with the cell membrane or phagocytosis [113]. Amongst the biologic functions of exosome, a role in modulation of immune response, as well as in antigen presenting, in coagulation and a singular role in spreading various pathogens like viruses and prions from one cell to another, have been reported [110].

A topic of particular interest is their function in horizontal transfer of genetic information [147]. It has been demonstrated that the miRNAs contained in exo-

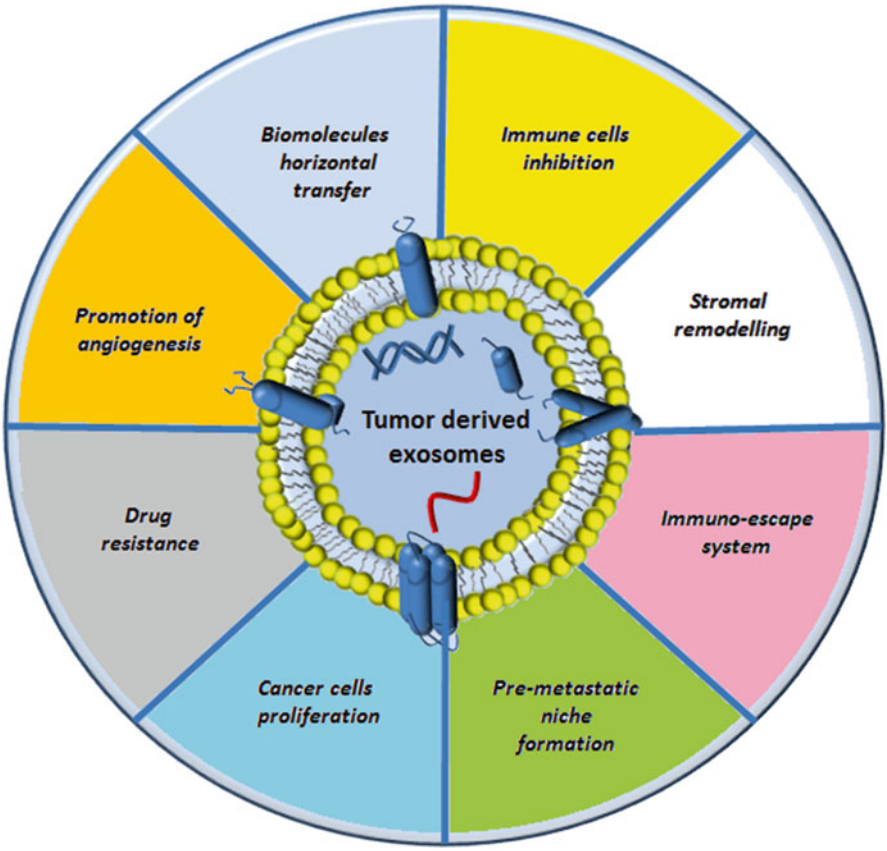


**Fig. 3** Different roles of Tumor derived exosomes

somes can be shuttled to other cells and modulate their genetic expression [126, 148–150].

## *6.3 Exosomes in Cancer*

In recent years, it has become clear that cancer cells release exosomes, and that this tumor-derived exosomes (TDEs) have pleiotropic functions in the disease (Fig. 3) [151].

It has been demonstrated that TDEs are involved in drug resistance mechanisms, by expelling hydrophilic drugs from cancer cells [152]. Moreover, it has been described that TDEs promote carcinogenesis, tumor growth, angiogenesis and extracellular matrix remodeling through secretion of matrix metalloproteinase (MMPs) or activators of MMPs [151].

TDEs have strong roles in the intercellular communication between cancer and stromal cells that modulate the tumor microenvironment and favor tumor growth [153]. In addition to effects exerted on the primary tumor microenvironment, TDEs also play a role in the establishment of the pre-metastatic niche [151].

In samples from melanoma patients, TDEs have been shown to promote metastasis by establishing metastatic niches through activation of bone marrow progenitor cells [30]. Interestingly, exosomal miRNAs have been associated with cancer, including lung cancer [154], ovary cancer [155] and glioblastoma [156]. It was also reported, both in *in vivo* and *in vitro* models of chronic myeloid leukemia (CML), that TDEs induce the release of interleukin-8 from epithelial cells, promoting an angiogenic phenotype in the tissue [157]. TDEs of metastatic breast cancer cells have been proven to contain several miRNA that induce angiogenesis, such as miRNA-210 [146]. Moreover, exosomes have been recently reported to undergo their own biogenesis of miRNA, promoting tumorigenesis and carcinogenesis [158]. This activity has recently highlighted exosomes as casual agents for the "seed and soil" hypothesis [159], which postulates that cancer disease educates the body tissues to acquire a pro-metastatic phenotype making it easier for CTCs to invade the tissue and cause metastasis [30, 160, 161].

## *6.4 The Role of TDEs in Cancer: From Biomarker Source, to Therapy Target or Potential Treatment Option*

The abnormal release of exosomes by tumor cells, as evidenced by their increased levels in blood during the disease, suggests a role of TDEs as useful tools for diagnostic and therapeutic studies. A topic of particular interest is that the proteins and miRNAs profile could be used as biomarkers for the early diagnosis and prognosis of disease, making TDEs suitable to use as a non-invasive diagnostic tool. As our knowledge and study of them is recent, exosomes are in preclinical or early clinical stage phase of development as biomarkers.

### 6.4.1 Exosome as Biomarkers

Several types of cancer TDEs components have been reported as biomarkers (Table 1):

One of the first reported analyses of TDEs in cancer discovered that 12 miRNA, upregulated in lung adenocarcinoma tissue samples, were also upregulated in TDEs present in the patients plasma samples, mirroring the conditions inside of the tumor cells [154]. In another report, the levels of let-7f and mir-30e-3p, present in the microvesicles of lung cancer patients, were capable of discrimination between patients of lung cancer for stage of disease, and also with a poor outcome [162]. Moreover, a very thorough trial have reported that exosomal miRNA signatures can

**Table 1** Summary of exosomal components reported as potential clinical biomarkers

| Disease | Source | Biomolecule | Biomolecules analyzed | Biomarker potential | Reference |
|---|---|---|---|---|---|
| Lung adenocarcinoma | Plasma | miRNA | miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, miR-214 | Diagnostic and prognostic: Detection of lung adenocarcinoma respect healthy patients | [154] |
| | | | miR-378a, miR-379, miR-139-5p, miR-200b-5p | Screening: Discrimination between nodule (LA+Carcinoma) and non-nodule (Healthy former smokers) | [163] |
| | | | miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, miR-154-3p | Diagnostic: Discriminate between lung adenocarcinoma and granuloma | |
| Lung Squamous Cell Carcinoma | Plasma | miRNA | miR-205, miR-19a, miR-19b, miR-30b, miR-20a | Diagnostic value: Presence related with presence/absence of tumor mass | [95] |
| Non-Small Cell Lung Cancer | Plasma | Protein | 30 protein markers, including exosome markers, known cancer markers and others (EV-Array). | Diagnostic and predictive: 30-Marker Panel had 75 % accuracy classification of patients later diagnosed with NSLC. | [131] |
| Esophageal Squamous Cell Carcinoma | Serum | miRNA | miR-21 | Diagnostic and prognostic | [164] |
| | | | miR-1246 | Diagnostic and prognostic | [165] |

(continued)

**Table 1** (continued)

| Disease | Source | Biomolecule | Biomolecules analyzed | Biomarker potential | Reference |
|---|---|---|---|---|---|
| Prostate cancer | Plasma | miRNA | miR-141, miR-375 | Diagnosis of metastasis and poor prognosis predictor. | [166] |
| | | | miR-1290, miR-375 | Prognostic biomarkers for castration-resistant prostate cancer patients | [145] |
| | | Protein | PTEN | Diagnosis and discrimination between cancer and healthy patients | [167] |
| | | | Survivin | Early detection, diagnosis and monitoring | [168] |
| | Urine | mRNA | PC-3, TMPRSS2:ERG fusion gene | Diagnosis and follow-up | [169] |
| Ovarian cancer | Serum | miRNA | miR-21, miR-141, miR-200[a], miR-200b, miR-200c, miR-203, miR-205, miR-214 | Diagnosis and screening, discriminating between benign disease and malignant disease | [155] |
| | Whole blood | Protein | L1CAM, CD24, ADAM10, EMMPRIN | Diagnosis of ovarian cancer | [170] |
| Glioblastoma | Serum | mRNA | EGFRvIII | Follow-up: Detection of mutated EGFR form (EGFRvIII) | [156] |
| Glioma | Serum | Protein | EGFR, EGFRvIII, and TGF-β | Diagnostic: Present in the exosomes of glioma patients | [171] |

(continued)

**Table 1** (continued)

| Disease | Source | Biomolecule | Biomolecules analyzed | Biomarker potential | Reference |
|---|---|---|---|---|---|
| Breast cancer | Serum | miRNA | miR-200[a], miR-200c, miR-205 | Diagnostic: Upregulated in exosomes of breast cancer patients respect healthy women | [172] |
| | | | miR-373 | Prognostic: Association between increased exosomal levels of miR-373 with triple receptor-negative breast cancer | [173] |
| Bladder cancer | Urine | Protein | EDIL-3 | Diagnosis and therapy target | [174] |
| | | | TACSTD2 | Diagnosis of bladder cancer respect healthy controls | [175] |
| Melanoma | Plasma | Protein | CD63, Caveolin | Diagnosis: Exosomes containing this proteins were more abundant in melanoma patients respect healthy donors | [176] |
| | | | TYRP2, VLA-4, HSP70, HSP90, MET | Prognostic and therapeutic | [177] |

be used to perform initial screening of lung adenocarcinoma patients, and furthermore classify the patients with high sensitivity and specificity when compared with CT scan [163]. Recently, a panel of TDE proteins has been reported with significant signature expression between Stage III/IV NSCLC patients and healthy volunteers, which could be used as a tool for NSCLC screening [131]. Our group is currently performing analysis of miRNA signatures in TDEs of NSCLC patients with several mutations relevant to targeted therapy treatment, showing preliminary promising results regarding its utility as prognostic and predictive biomarkers [164]. In lung squamous cell carcinoma (SCC) patients, TDE miRNA signatures have been reported to be downregulated after resection of the tumor mass, to be present in the TDEs of the patients prior to tumor resection, and to be significantly lowered after the surgery as well [95], confirming its potential as diagnostic and follow-up biomarkers. In breast cancer, the detection of exosomal mir-373 in serum have been

correlated with triple negative breast cancer [165]. It is also interesting to note that differences between the levels of this miRNA as a serum-free form and the levels inside the exosomes have been established. In ovarian cancer, several miRNA signatures have been identified, with a value as diagnostic biomarkers [155]. Also in the serum of ovarian cancer patients, different profiles between proteins and miRNAs in TDEs compared to the exosomes of cancer-free individuals [166] have been shown to be useful to define a "barcode" to screen for ovarian cancer patients. In prostate cancer, exosomal mir-1290 and mir-375 in serum have been demonstrated to have a prognostic value in prostate cancer patients [145]. It has also been revealed that the detection of the survivin protein in exosomes could be used for early detection of prostate cancer [167].

### 6.4.2 Exosomes as Therapy

Several studies suggest that TDEs may be involved in different mechanisms of tumor resistance, such as in drug resistance and in resistance to radiotherapy [160, 168–170]. Thus, extract the TDEs could have a benefit on the patient. In this way, one approach that has been reported is the development of a selective hemodialysis-like filter to remove TDEs from the blood circulation of patients [171]. This approach has the potential to sensitize tumor cells to treatments or to overcome the immunosuppression induced by the exosomes [171].

Other studies, acknowledging that the uptake of TDEs is a specific process, have explored the potential of repurpose the TDEs to be used as vaccines against cancer [172, 173]. Phase I clinical trials have been performed using dendritic-cell derived exosomes recovered from ascites but, unfortunately, only a few patients received benefits by stabilization of the disease [174]. A phase II clinical trial demonstrated that dendritic cell derived exosomes, carrying NKg2D ligands in association with T-reg cell inhibition treatments, were capable to induce a MHC response and a tumor regression in melanoma patients [175]. In NSCLC, a phase I study was performed using autologous dendritic cell derived exosomes (Dex) from the enrolled patients, loaded with MAGE antigens, as immunotherapy [176]. Another phase II clinical trial, started in 2010 (NCT01159288), is currently recruiting for NSCLC patients to be treated with a combination of Dex loaded with tumor antigen and vaccination with metronomic cyclophosphamide, although no results have been published yet.

### 6.4.3 Exosome as Drug Delivery Tool

The hypothesis to use exosomes as specific targeted drug delivery systems of miRNAs, siRNAs or several drugs is a recent and attractive field of investigation, having potential to surpass several limits of engineered nanovesicles, such as the specific targeting of chemotherapy drugs or the induction of an immunogenic response [173].

Álvarez-Erviti et al. reported for the first time an electroporation method capable to introduce short-interference RNA (siRNA) into dendritic cell derived-exosomes [177], which then migrate and deliver their content to specific brain target cells in a mice model. These reports showed that exosomes were capable to deliver a treatment that overcomes the blood brain barrier without triggering an immune response [178]. Similar experiments have been reported in breast cancer mouse models with the introduction and delivery of doxorubicin loaded in TDEs through electroporation [179]. The successful loading of exosomes with let-7a miRNA and its selected delivery to breast cancer cells in mice xenograft models has been recently shown [180].

## 7 Concluding Remarks

In this chapter we have addressed the issue of how the latest discoveries in cancer have opened new challenges in order to improve the possibilities for our patients, not only in therapy, but also in follow up, and when possible, in prevention. Early diagnosis, molecular tumor profiling and targeted therapies require new biomarkers, which are representative for the tumor heterogeneity, available to be analyzed continuously and obtained in a non-invasive fashion. We have described the relatively recent arise of the liquid biopsies as an answer to these challenges, unraveling new entities involved in the disease development. Through this chapter, we have discussed briefly every component discovered so far, and described their promise of a great variety of resources for the development of new diagnostic, prognostic, follow-up, prevention and even new therapeutic tools.

However, the promising preliminary results in clinical practice have some drawbacks that need to be solved before taking them into daily clinical practice.

A common issue to all the liquid biopsy components is that their isolation and analysis require very specific procedures and equipment, which are not always feasible for clinical practice. The standardization of the methods in order to guarantee reproducibility is mandatory. Besides, there are several problems specific for each component of the liquid biopsies.

Their low numbers in initial stages, as well as their known capability to undergo phenotype EMT-MET transitions, difficults the detection of CTCs. By adding that the only approved method by the Food and Drug Administration (FDA) detects CTCs with an epithelial phenotype, and the great variability of CTC secretion between patients, the problems of CTCs analysis become more evident.

In the field of ctDNA analysis there are major problems regarding the discrimination of the ctDNA amongst the background of cfDNA coming from other sources (autoimmune process or traumas), hampering severely its utility as diagnostic biomarker both in early stage and in late disease. Their utility for screening of tumor related mutations is also compromised due to the lack of standardization of isolation techniques, which are critical to avoid the contamination from cfDNA of the different cell types that are present in blood. Moreover, secretion of ctDNA into the blood stream represents a great intra-patient variability, even in groups of patients showing a similar histology. Overall, there is a need for clinical trials that analyze the

biomarker role of ctDNA with clear distinction of the analyzed population, for example, analyzing the molecular alterations on one defined disease stage, with a sufficient number of participants to overcome patient intra-variability and establish significant correlations.

Because cfmiRNAs and exosomes were the last components discovered, their role in the liquid biopsy context is largely unknown. Their isolation and analysis techniques are still in the preliminary stages, with ongoing discussion about the best approach for their isolation and analysis. In the exosomes specifically, the techniques to discriminate between the different subpopulations secreted by tumor cells are challenging, making it difficult to accept analysis and validation as biomarkers.

Nevertheless we think that these obstacles will be surpassed soon. Actually, great efforts are being carried out by the scientific community to develop new technologies of isolation and analysis of liquid biopsy components. Moreover, several clinical trials are evaluating components of the liquid biopsies as diagnostic and prognostic tools to provide the scientific background and statistical strength to their use as biomarkers, and also combining them, as it has happened already with joint analysis of CTCs and ctDNA.

If this trend continues, we think that each component of the liquid biopsies will have soon its own niche in the field of oncology. As NGS analysis is combined with more sensitive isolation of CTCs, based on detection of new immunological affinity targets, independent of an epithelial phenotype and physical properties, we suggest that the CTCs will be useful in the detection and monitoring of cancer types with early stage metastasis. As the new isolation and analysis techniques are capable to discriminate between tumor and non-tumor ctDNA [181], it will allow for its routine monitoring as a guide for target identification, targeted therapy choice and follow-up of effectiveness. Finally, the cfmiRNA and exosome profiles, despite that they are behind in terms of their application, they are cutting ground quickly, as it is shown for the exponential increase in publications with exosomes as main topic [141]. All this new knowledge boosts the development of techniques for standardized isolation and analysis of exosomes [132, 182]. At the same time, new techniques for multiplex profile of miRNA involved in various cancer types, both cell-free and exosomal, and the analysis of exosomal proteins, have become recently available. Once these techniques are tested, we are confident that the cfmiRNA and exosome profiles will be used soon in the clinic to describe in detail the early disease stage tumor heterogeneity, and that the exosomes will bring a revolution to cancer treatment with their role as highly specific vehicles for targeted therapies.

Overall, the liquid biopsies promise great advances, present stimulant challenges and are, in our opinion, the best option to develop biomarkers and therapies that are up to the ideal of the personalized medicine.

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# Targeting Tumor Angiogenesis for Cancer Prevention

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## 1 Introduction

Cancer is a major public health problem in the world, currently reaching heart diseases as the leading cause of death in developed countries. So, just in the USA the lifetime probability of being diagnosed with an invasive cancer is 43 % for men and 38 % for women, with more than 4500 new cancer diagnoses each day [1]. Very often, localized tumors can be successfully treated by surgery, radiotherapy and other methods. On the contrary, medicine is often helpless in the face of metastatic cancer. Current cancer research mainly focuses on the identification of specific agents with a potential to either suppress tumor growth or prevent the processes allowing benign tumors to gain metastatic competence and begin to spread to distant organs [2].

Tumor progression involves the stepwise accumulation of both genotypic and phenotypic alterations, resulting from generalized carcinogen exposure as well as clonal proliferation of mutated cells. The arrest of one or several of the steps of tumor progression may impede or delay the development of cancer. Cancer chemoprevention, as firstly defined by Sporn in 1976, makes use of natural, synthetic, or biologic agents to reverse, suppress, or prevent tumor progression [3].

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Cancer prevention can be applied at any stage along the natural history of cancer disease, with the objective of halting further progression of the condition. Cancer preventive actions may be implemented at several levels [4]:

(a) Primary prevention, frequently applied to broad populations, is directed to reduce the incidence of disease either by reducing exposure to carcinogenic factors or by increasing the individuals' resistance to them.
(b) Secondary prevention, targeting more closely defined higher-risk populations, is focused to reduce mortality from a particular cancer through detection and treatment in its earliest stages, before symptoms appear.
(c) Tertiary prevention is aimed at improving the survival rates and the quality of life of cancer patients. It also includes the prevention of recurrence of a preexisting cancer or the development of new secondary tumors.

Prevention is probably the most cost-effective long-term strategy for the control of cancer. For many patients cancer becomes a chronic disorder that has to be treated for a long period of time. In this case, therapeutic strategies should be selected in order to minimize their long-term toxicities. On the other hand, they should also take into account cost issues that could make prohibitive their application to the vast majority of the patients. In this regard, low cost natural drugs, such as phytochemicals, could be a valuable source of chemopreventive agents used either to reverse or to slow down the cancer progression [5].

In spite of the great efforts made in search of cancer chemoprevention strategies in the past three decades, these strategies have yet to find clinical application on a large scale [6]. Advances in basic research, crucial to unveil the molecular mechanisms in carcinogenesis, will provide the basis for the identification and development of therapeutic strategies to either prevent the occurrence of precancerous lesions or to delay their progression to invasive disease. In this regard, a description of the main hallmarks of cancer has contributed to set a conceptual framework that can be used to understand the complexity of the biology of cancer, and to design and develop future molecular targeted therapies and strategies for cancer prevention, based on modulation of one or several hallmarks of cancer.

## 2 The Hallmarks of Cancer

There are over 200 different types of cancers that affect virtually every organ. In spite of the extreme diversity of oncologic diseases, they share some fundamental features, pointed out by Hanahan and Weinberg in their seminal article published in 2000 [7] and revisited in [8]. They will be briefly detailed below:

### 2.1 Self-Sufficiency in Relation to Growth Signals

Tumor cells show a reduced dependence on external stimulating signals from their microenvironment. This may be achieved by different ways including increases in the number of surface receptors, structural alterations in the receptor molecules that facilitate ligand-independent firing, self-generation of growth factors or activation of normal cells in the tumor-microenvironment, among others [9, 10]. Given that deregulated proliferation is one of the major characteristic of tumorigenesis, it is considered to be one of the main targets for chemoprevention [11].

### 2.2 Insensitivity to Growth Suppressors

In normal conditions, antiproliferative factors are produced to maintain cellular quiescence and tissue homeostasis. Tumor suppressor genes, such as retinoblastoma-associated (RB) and p53 proteins, play key roles in the mechanisms that help cells to decide between their proliferation and their senescence and apoptotic way. Inactivation of tumor suppressor genes in cancer cells allow them to evade the programs that exert a negative regulation on cell progression in order to grow uncontrollably [12–14]. The coordination of anti-growth signaling and natural compound studies will provide insight into the future application of these compounds in chemoprevention of cancer [15].

### 2.3 Evasion of Cell Death Mechanisms

"Apoptosis" is a physiological process of programmed cell death that occurs throughout life during development and plays a critical role in maintaining cellular homeostasis [16]. Tumor cells prevent their self-destruction circumventing normal apoptotic mechanisms by several means: the loss of p53 tumor suppressor function, the upregulated expression of Bcl-2, Bcl-xL, the downregulated expression of Bax, Bim and Puma, and a significant loss or inactivation of lead members in the caspase family, among others [17–19]. A number of nutraceuticals, including curcumin, flavopiridol, genistein and resveratrol, among many others, have shown potential to decrease tumor cell survival by activation of the mechanisms of programmed cell death [11, 20].

### 2.4 Unlimited Replicative Potential

Normal cells have a limited number of cell division cycles associated with two different processes that block cell proliferation: senescence (an irreversible, viable but quiescent, unproliferative state) and crisis (a state that induces cell

death). The replicative potential of cells is related to the telomeres, regions of repetitive nucleotide sequences located at the end of each chromatid, which are shortened during each cell generation. Telomerase, the DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in non-immortalized or somatic cells, but expressed in the immortalized cells, allowing them to maintain their telomere length and proliferative potential. The acquisition of telomerase function serves to generate tumor-promoting genomic alterations and its subsequent activation stabilizes the mutations and confers the unlimited replicative capacity that cancer cells require in order to generate clinically apparent tumors [21–23]. Down regulation of telomerase activity has been proposed to contribute to the potential chemopreventive effect of some nutraceuticals [24, 25].

## *2.5 Angiogenesis Induction*

During embryogenesis, the development of the vasculature involves the creation of new blood vessels from endothelial precursors by a process termed vasculogenesis. Once the primary vascular network is formed the sprouting of new blood vessels from pre-existing ones occurs by angiogenesis [26]. Angiogenesis is a highly regulated process, very active in embryos but largely quiescent in adults, limited to some processes related to reproductive cycles, wound healing and bone repair. Nevertheless, a deregulated and persistently activated angiogenesis is essential for tumor growth and metastasis, facilitating their sustenance through the availability of nutrients and oxygen, as well as the removal of metabolic wastes and carbon dioxide [27]. The molecular mechanisms controlling tumor angiogenesis and the potential of the use of antiangiogenic compounds as chemopreventive agents will be discussed in more detail in the next sections of this chapter.

## *2.6 Invasion and Metastasis*

The invasion-metastasis cascade is the result of a series of subsequent discrete steps: local invasion, intravasation by cancer cells into blood and lymphatic vessels, pass of cancer cells through the blood and lymphatic systems, extravasation of cancer cells into the parenchyma of distant tissues, formation of small nodules of cancer cells, and finally, growth of micrometastatic lesions into macroscopic tumors. Metastasis involves interactions between cancer cells and the local microenvironment. Alterations in molecules involved in the cell-to-cell and cell-to-extracellular matrix adhesion promote the invasion-metastasis cascade steps [28, 29]. A wide variety of nutraceuticals derived from natural sources inhibit tumor cell invasion and metastasis by targeting one or several steps of the invasion-metastasis cascade [11].

## 2.7 Genome Instability and Mutations

Genomic instability is a characteristic that is causally associated with the acquisition of hallmark capabilities by cancer cells. Defects in genome maintenance and repair systems are selectively favorable and instrumental for tumor progression. Genomic instability has been specially attributed to the mutations in "caretaker" genes, whose primary function is to maintain genomic stability. Classical caretaker genes are DNA repair genes and mitotic checkpoint genes, although p53 could also be considered as a caretaker gene because of its function in the DNA damage response. Either through inactivating mutations or via epigenetic repression the caretaker genes can lose their properties as tumor suppressor genes in the course of tumor progression, or this will accelerate the rate at which evolving premalignant cells can accumulate advantaged genotypes [30–33].

## 2.8 Tumor-Promoting Inflammation

Complex interactions between neoplastic cells, non-malignant stromal cells, and migratory hematopoietic cells, including cells from the innate and adaptive immune system regulate tumor growth, progression, angiogenesis and metastasis [34, 35]. In some cancers, inflammatory conditions precede development of malignancy; in others, oncogenic change drives a tumor-promoting inflammatory milieu filled by tumor-promoting inflammatory cells including macrophage subtypes, mast cells, and neutrophils, as well as T and B lymphocytes [36–39]. These cells also stimulate angiogenesis and tumor progression [40]. In contrast to the transient immune inflammation arising after a normal lesion or infection, a persistent inflammatory status is associated with tumor progression [41]. A persistent long term activation of NFκB, a central regulator of innate immune response, is tumorigenic. It has also been shown that a down regulation of NFκB activity directly, or indirectly through the activation of the p53 pathway reduces tumor growth substantially. Efforts are already underway to find chemopreventive compounds that will restore to normal the activities of this inflammatory mediator to regress tumor progression as well as its aggressiveness [11, 42].

## 2.9 Evasion of Immune Destruction

The immune system is crucial to resist or eradicate the formation and progression of incipient neoplasias, late-stage tumors, and micrometastases. Tumor cells are characterized by the expression of specific antigens that distinguish them from their normal counterparts, making them recognizable by an ever-alert immune system. Following this reasonable cycle of responses, nobody would develop tumors [43].

However, cancer cells can evade immune destruction by disabling immunological components that have been designed to eliminate them. Solid tumors appear because either they can avoid their detection by the various arms of the immune system, or they are able to limit the extent of immunological killing, thereby evading eradication [44, 45]. Many chemopreventive agents including aspirin and other cyclooxygenase (COX)-2 inhibitors, aromatase inhibitors, and bisphosphonates can mediate their effects, at least in part, by reversing the mechanisms used by cancer cells to escape from immune destruction [46].

## *2.10 Energy Metabolism Reprogramming*

Tumor cells are characterized by a significant activation of glycolysis, so that they predominantly produce ATP/energy through the glycolytic pathway rather than through the tricarboxylic acid cycle, even in the presence of adequate oxygen, what is known as "Warburg Effect" [47]. In fact, not only glucose metabolism is changed in cancer but also growing evidence indicate that cancer cells undergo an overall metabolic reprogramming, also affecting glutamine and lipid metabolism [48, 49]. One challenge for chemoprevention could be the reprogramming of the altered metabolism of a cancer cell toward that of un-transformed cell. In this sense, it has been described that metformin, an oral antidiabetic drug, could be effective to reset the metabolism of cancer cells even in those subpopulations exhibiting cancer stem like features [50].

Chemoprevention may target several steps in tumor initiation, promotion and progression. Likely, many chemopreventive agents will have effect throughout the carcinogenic process by interfering more than one hallmarks of cancer [51]. This is the case of the tight relation of inflammation and angiogenesis being reinforced by evidence showing that several anti-inflammatory compounds also prevent angiogenesis. This mechanism of "indirect" angiogenesis set it as a target of therapy and, even better, as a target of prevention of tumor angiogenesis by anti-inflammatory agents. Ideally, a chemopreventive anti-inflammatory approach will be able to block neovascularization before the connection of the angiogenic switch, resulting in a significant delay in the appearance of a clinically relevant cancer. Clinical data suggest that the regular use of nonsteroidal anti-inflammatory drugs (NSAID) is associated with reduced risk of some cancers [52]. This family of drugs fall under the sunshade of angiopreventive molecules not only for their innate immune cell suppression, but also by inhibition of COX enzymes, which are upstream of vascular endothelial growth factor (VEGF) production by stromal cells, as well as for the hepatocyte growth factor (HGF) [53]. This example can also help to illustrate the huge amount of work yet to be done for cancer prevention to mirror the success obtained in cardiovascular medicine. Having been aspirin, a COX inhibitor, recognized as one of the agents with a high potential in colorectal cancer prevention, this has not been yet assessed in randomized trials in this disease [51].

## 3 Angiogenesis and Cancer

### *3.1 A Persistent and Deregulated Angiogenesis Is a Hallmark of Cancer*

Blood vessels form the largest network in our body, being responsible for the supply of nutrients to the whole organism. Angiogenesis is a highly regulated process, very active in embryos but largely quiescent in adults, limited to some processes such as wound healing, bone repair and blood flow restoration after an insult or injury. In females, angiogenesis is also present during the reproductive cycle (*corpus luteum* formation, endometrial vascularization) and during pregnancy (placental development) [54]. The normal healthy body maintains a perfect balance of angiogenesis modulators leading to an inhibition of this process. Nevertheless, when angiogenic growth factors are produced in excess of angiogenic inhibitors, the connection of the so called "angiogenesis switch" takes place. After activation, endothelial cells undergo a series of phenotypic changes, including the release of proteases facilitating them to degrade the extracellular matrix, migrate, proliferate, avoid apoptosis, and, finally, differentiate to form new vessels. Once the new sprouts are formed they become stabilized by mural cells (pericytes and smooth muscle cells) in a process that is critical for the new vasculature to become stable, mature and functional.

Breakdown of the extracellular matrix is mediated by several proteinase families and their inhibitors, including those of plasminogen activators, matrix metalloproteinases (MMPs), and cathepsins, among others. Proteolytic degradation of extracellular matrix molecules is an integral part of angiogenesis, as it does not only provide scaffold support to migrating endothelial cells but also results in liberating matrix-bound angiogenic growth factors [55–58].

A deregulated and persistent activation of the angiogenic switch is related to an increasing number of inflammatory, allergic, infectious, traumatic, metabolic or hormonal disorders, characterized by an excessive vessel growth related to an upregulated angiogenesis [59]. They include cancer, proliferative retinopathies, macular degeneration, psoriasis and rheumatoid arthritis. Progress in understanding the process of angiogenesis, the isolation of angiogenic growth factors, the successful preclinical studies and the promising results from clinical trials have created great excitement about the potential of therapeutic angiogenesis and antiangiogenic therapies for many diseases, including cancer [27, 60].

The use of inhibitors of angiogenesis was firstly proposed by Judah Folkman as a new cancer therapy over 40 years ago [61]. The fact that this new therapeutic strategy was focused to the activated endothelial cells, responsible for the formation of new blood vessels, would make it applicable to a wide variety of tumors. Moreover, because of low mutagenic potential of endothelial cells, tumors should not develop resistance to the effects of many of these inhibitors [62, 63]. The connection of the angiogenic switch can occur at any stage of tumor progression, depending on both the type of tumor and its microenvironment (Fig. 1). Many tumors start growing in an avascular phase until they reach a steady state level of proliferating cells. At this

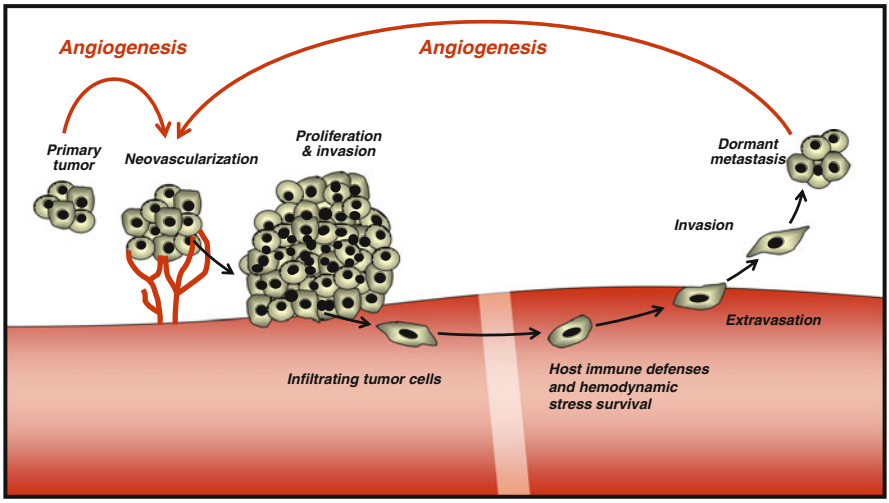


**Fig. 1** Angioprevention of cancer. The formation of new blood vessels is essential for tumor to grow. The acquisition of an angiogenic phenotype also facilitates tumor cells to gain access to blood circulation, crossing vessel walls. Those infiltrating tumor cells surviving immune defense and hemodynamic stress could undergo extravasation, giving rise to silent metastatic colonies in a phenomenon called dormancy. After a second phase of uncontrolled proliferation and angiogenesis activation will trigger the growth of clinically detectable secondary tumors. Antiangiogenic molecules can be used to prevent the activation of the angiogenic switch in the early steps of tumor progression as well as in the micrometastasis awakening from their dormant state

point, angiogenesis is activated to ensure exponential tumor growth starting with perivascular detachment and vessel dilation followed by angiogenic sprouting, new vessel formation and the recruitment of perivascular cells [64]. The newly formed vessels also provide a pathway for tumor cells to evade the primary tumor and colonize at secondary sites. There, the colony of metastatic cells could remain in a clinically undetectable "dormant" state, until the moment when angiogenic switch is activated allowing micrometastasis to grow and give rise to secondary tumors.

Tumor cells may overexpress one or more of the positive regulators of angiogenesis, mobilize an angiogenic protein from the ECM, recruit host cells such as macrophages (which produce their own angiogenic proteins), or engage a combination of all these processes. Tumor vasculature differs significantly from normal vasculature. Tumor-vessels are usually disorganized, often incomplete, lacking structural integrity and prone to collapse, resulting in areas of inadequate perfusion and transient hypoxia. The tumor pericytes coverage shows a lower density, with looser connection between pericytes and endothelial cells. The aberrant tumor vasculature is often disorganized and incomplete, showing abnormal blood flow and increased permeability, and lacking structural integrity, what causes local areas of inadequate perfusion and transient hypoxia [65].

Although many questions remain unanswered, modulation of therapeutic angiogenesis may be the next major advance in the treatment of an increasing number of

pathologies where an angiogenic disorder is indeed deeply involved in the origin of the illness and/or its progression. Although the first results arising from the clinical setting made doubt about the real potential of inhibition of angiogenesis for the treatment of cancer, nowadays the number of antiangiogenic drugs and treatment being approved for their clinical use is continuously growing [66]. The accumulating clinical evidences of antiangiogenic therapies in extending survival in patients with advanced cancers and supplying new strategies for the treatment of blindness and other angiogenesis-dependent pathologies has propelled the interest in the clinical development of angiogenesis inhibitors, making of antiangiogenesis one of the more active fields in Pharmacology.

## *3.2 Hypoxia Is One of the Main Stimulus for Tumor Angiogenesis*

As mentioned above, the control of the angiogenesis switch is achieved by a tight balance between positive and negative regulatory molecules. Initial stages of tumor growth lead to hypoxic regions inside the tumor, promoting tumor and stromal cell secretion of potent proangiogenic growth factors via the activation of the Hypoxia Inducible Factors (HIFs). Upregulation of the HIF system is observed in many common cancers and occurs by a multiplicity of genetic and environmental mechanisms. Besides the activation by hypoxia, HIF-induced angiogenesis can also be induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways, as well as after the inactivation of tumor-supressor genes, indicating that HIF activation can be considered a consequence of the oncogenic malignization process. Among the known transduction pathways mediating responses to these stimuli, both, the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol (PI)-3 kinase pathways have been implicated in these processes [67, 68].

HIFs are a family of heterodimeric transcription factors composed of alpha and beta subunits. The beta subunit is constitutively expressed, while the alpha subunit is tightly oxygen-regulated, so that regulation of HIF activity is mediated primarily through the stability of the alpha subunit: in the presence of oxygen, HIF-α proteins are translated but rapidly degraded. Nevertheless, in hypoxia HIF-α proteins stabilize, accumulate, and migrate to the nucleus, associate with beta subunits and form the HIF-1 and HIF-2 heterodimers. HIF mediate transcriptional responses and can promote tumor progression by altering cellular metabolism [69, 70]. By binding to specific HRE (hypoxia response elements) in their promoters, these heterodimers may induce the expression of at least 150 genes encoding proteins that regulate cell metabolism, survival, motility, basement membrane integrity, angiogenesis, hematopoiesis, and other functions. Some relevant activators of angiogenesis, including VEGF are among these genes [71]. In such a situation, HIF activates the angiogenic switch on surrounding vessels in

order to promote blood vessel formation towards the hypoxic focus and to alleviate oxygen insufficiency. Efforts are currently under way to develop targeted cancer therapeutics to hypoxia-activated pathways, and in particular to the search for HIF-1 inhibitors for the treatment or prevention of cancer [71–73].

### *3.3 Molecular Mechanisms of Tumor Angiogenesis*

The connection of the angiogenesis switch involves the activation of very diverse intracellular signaling pathways [74]. Those pathways are related and interconnected, being redundant in some cases. A comprehensive understanding of the molecular mechanism of angiogenesis will result in the design of more effective therapeutic strategies.

Among the growth factors involved in the activation of tumor angiogenesis, the isoform A of VEGF plays the most relevant role [75, 76]. It promotes proliferation, migration, differentiation and survival of the endothelial cells, as well as the activation of the mechanisms of extracellular matrix degradation and vascular permeability. VEGF-A expression is regulated by multiple factors, including cellular hypoxia, as well as a number of extracellular signals such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF)-1 and several other cytokines [77].

Binding of VEGF-A to the receptor VEGFR2 leads to receptor dimerization and autophosphorylation of the intracytoplasmic domains in specific tyrosine residues located in the carboxy-terminal region, what activates a tyrosine kinase cascade involving various intracellular proteins, particularly the PI-3 kinase [78]. VEGFR2-induced pathways include the proliferative pathway, mediated by the extracellular signal regulated kinase (ERK)-MAPK cascade, and the AKT/PKB pathway, involved in both, the control of cellular survival by inhibiting pro-apoptotic pathways, and the activation of endothelial nitric oxide synthase (eNOS), implicated in the increase in vascular permeability and cellular migration. VEGFR2 signaling is also modulated through neuropilins, which act as VEGF co-receptors, by the receptor VEGFR1, and by heparan sulfate and integrins [77].

Clinical evidence supports the relevance of the angiopoietin-Tie system in the control of the tumor angiogenesis switch. The opposing effects of the angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) binding with their specific receptor Tie2 play a crucial role in the regulation of the angiogenic remodelling and vessel stabilization that take place after VEGF action [79]. Ang-1, secreted by endothelial support cells, activates Tie2 by tyrosine phosphorylation, what maintains the endothelium in a mature quiescent state by facilitating recruitment and high association with mural cells and mediating survival signals for endothelial cells. Localised expression of Ang-2, which inhibits the Ang-1 mediated Tie2 activation, primes the vascular endothelium to exogenous cytokines, such as VEGF,

allowing vascular remodelling [80]. In the absence of the mitogenic signal from VEGF, however, endothelial cells are more likely to undergo apoptosis, leading to vessel regression [81].

Some other factors, such as the members of the FGF family, activate a broad range of target cells besides endothelial cells. FGF signaling, mediated by FGFRs, follows a classic receptor tyrosine kinase signaling pathway and plays a key role in the maintenance of vascular integrity [82]. A deregulation at various points of the FGF cascade results in malignancy. Moreover, activation of FGF signaling pathways may be related to the appearance of resistance to anti-VEGFR2 therapies, involving vascular regrowth in a VEGF-independent second wave of angiogenesis [83].

HGF is a protein secreted by fibroblasts and perivascular cells that also exhibits angiogenic activity *in vivo* and *in vitro* [84]. The HGF receptor is encoded by the proto-oncogen c-Met, a tyrosine-kinase receptor mainly expressed by epithelia and also present in the endothelium, where it is induced by hypoxia. The proliferative response induced in endothelial cells by HGF involves activation of the ERK-MAPK pathway, whereas the antiapoptotic effect of the HGF/c-Met axis is mediated by PI3K/Akt pathway [85, 86]. Since c-Met is frequently expressed in cancer, its activation resulting in increased proliferation, invasion and survival of cancer cells, it is an interesting target for inhibition of both, tumor growth and tumor angiogenesis [86].

Notch signaling is also important to determine how an endothelial cell responds to VEGF regulating multiple components of the VEGFR system. During the sprouting process, endothelial cells must undergo a functional specialization into "tip cells" that follow guidance signals and migrate extensively into avascular tissues, and proliferating "stalk cells" that must stay connected to the parent blood vessel [87]. Such organization is under control of the Dll4 (Delta-like 4)/Notch signaling pathway, which sets a hierarchy in receptiveness of cells to VEGF-A. During sprouting angiogenesis, tip cell formation is the default response to VEGF, whereas the stalk cell phenotype is acquired through Dll4/Notch-mediated lateral inhibition [88]. Notch signaling determines how endothelial cells respond to VEGF, which, in turn, regulates the expression of both, Notch and Dll4. The interplay between VEGF and Dll4/Notch signaling, critical for the angiogenic process, opens exciting possibilities for combined therapies simultaneously targeting both pathways [89–91].

Although endothelial cells are the main responsible of the sprouting of blood vessels, other cell types are involved in angiogenesis modulation. A wide variety of stroma cells, including fibroblasts, immune and inflammatory cells infiltrating the tumor, contribute to the modulation of angiogenesis by production of angiogenesis activators, inhibitors and chemokines [92–94]. Pericyte recruitment, in response to growth factors such as PDGF is essential for the maturation, remodelling and maintenance of the vascular system [95]. Pericyte-mediated survival signals confer resistance to VEGF antagonists, what explains the observation that VEGF-targeting therapies are mostly active on immature vessels lacking pericyte coverage and advises the use of multitargeted approaches to reach an effective inhibition of tumor angiogenesis [96].

### 3.4 Inhibitors of Angiogenesis: Better in Combination

The role played by VEGF-A in the control of the tumor angiogenic switch has been determinant in the development of antiangiogenic drugs most of them designed to neutralize the activation of endothelial cells by this angiogenic factor, either by blocking VEGF-A or by inhibiting the activation of VEGFR2 [66]. The most successful approach to block VEGF has been achieved by the use of humanized neutralizing antibodies (Genentech/Roche's bevacizumab®), firstly approved in 2004 for use in combination with fluorouracil-based chemotherapy as first-line treatment for metastatic cancer of the colon or rectum. Since then, bevacizumab indications have extended to many other types of tumors, including non-small cell lung cancer, breast cancer, glioblastoma or renal cell carcinoma, but always in combination with standard chemotherapy. Besides bevacizumab, other anti-VEGF treatments have been approved for cancer patients, including the use of drugs that are focused to inhibit the VEGFR2 activation. This is the case of an increasing number of low molecular weight tyrosine kinase inhibitors, including sunitinib, sorafenib, pazopanib and axitinib, being approved for the treatment of cancer patients [97]. The success of these multikinase inhibitors confirms the convenience of targeting simultaneously several cell types in the tumor environment, namely pericytes, tumor and endothelial cells, by combined inhibition of the PDGF, VEGF and oncogenes signaling pathways [98].

Yet a critical review of these results has shown up several limitations of these therapeutic strategies: the success of a given antiangiogenic therapy depends on the type of tumors, and although in some cases VEGFR inhibitors seem to work as monotherapies, no survival benefit has been obtained in most of the anti-VEGF monotherapy trials [99]. The limited clinical success met by antiangiogenic monotherapies could be explained by the high complexity of angiogenesis regulation, exerted by a complex network of pro and antiangiogenic factors [74]. In this sense, multidrug approaches, acting simultaneously on several pathways and/or type of cells, could be expected to render better results than monotherapy. Results obtained so far in clinical trials confirm this hypothesis and show that cytotoxic agents or radiotherapy in combination with inhibitors of angiogenesis exhibit at least additive, if not synergistic, antitumor effects. In addition, the use of combined therapies could help to overcome the limitations of each leading to enhanced efficacy and diminished toxicity.

Although antiangiogenic therapy was initially thought to be "a treatment resistant to resistance", clinical experience indicates that resistance to antiangiogenic drugs is unavoidable, with both patients whose tumors are intrinsically resistant to such drugs and those others that develop acquired resistance during the antiangiogenic treatment. The fact that angiogenesis differs among tumor types is in the basis of the intrinsic resistance to antiangiogenic therapies, whereas potential mechanisms of acquired resistance to antiangiogenic agents include up-regulation of alternative proangiogenic signals, increased production of proangiogenic factors by stromal cells, activation of an invasive phenotype or induction of alternative mechanism of

vascularization [100]. Strategies to overcome the resistance to antiangiogenic therapy include the combination of antiangiogenic agents with standard chemotherapy regimens, or other biologically targeted agents, and the use of standard therapies with antiangiogenic intent [101]. The high complexity of tumor angiogenesis provides a strong biological rationale for therapies that block multiple angiogenic pathways simultaneously to overcome resistance and produce a greater degree of regression and more durable responses.

## 4 Potential of Inhibitors of Angiogenesis in Cancer Prevention

Inherent to the Folkman's visionary perception that the formation of new blood vessels was required for tumor to grow was the proposal of the use of inhibitors of angiogenesis as chemopreventive agents able to stop the growth of both early primary tumor and metastasis [61]. Three decades later, the term "angioprevention" was coined by Adriana Albini's group, after the observation that many chemopreventive molecules were also inhibitors of angiogenesis, also suggesting that many of the inhibitor of angiogenesis designed for anticancer chemotherapy could be useful for cancer prevention [102]. Following this rationale, dietary inhibitors of angiogenesis can be potential drug candidates for the angioprevention of cancer.

Medicinal herbs, vegetables and fruits have been demonstrated to be a valuable source of inhibitors of angiogenesis that could be used as chemopreventive agents. These nutraceuticals, defined as parts of a food that provide medical and health benefits, should be expected to be less expensive, safer and more available than synthetic antiangiogenic drugs, what could facilitate their widespread use in the long term schedules required for an effective chemoprevention of cancer. Although specialized diets are not the only way to decrease the chances to develop cancer, those that are enriched in antiangiogenic molecules could be used to prevent the activation of the angiogenic switch in the early steps of tumor progression as well as in the micrometastasis awakening from their dormant state. Some of the main inhibitors of angiogenesis from dietary sources will be reviewed in this chapter.

### *4.1 Green Tea Catechins*

Growing evidence shows that green tea consumption is associated with reduced risk of cancer. Green tea polyphenols, most of them belonging to the catechins family, show chemopreventive and antiangiogenic activity [103]. Epigallocatechin-3-gallate (EGCG) (Fig. 2), probably responsible for much of the cancer chemopreventive properties of green tea, is powerful antioxidant, as well as an antiangiogenic, antiinflammatory and antitumor agent capable to modulate tumor cell response to

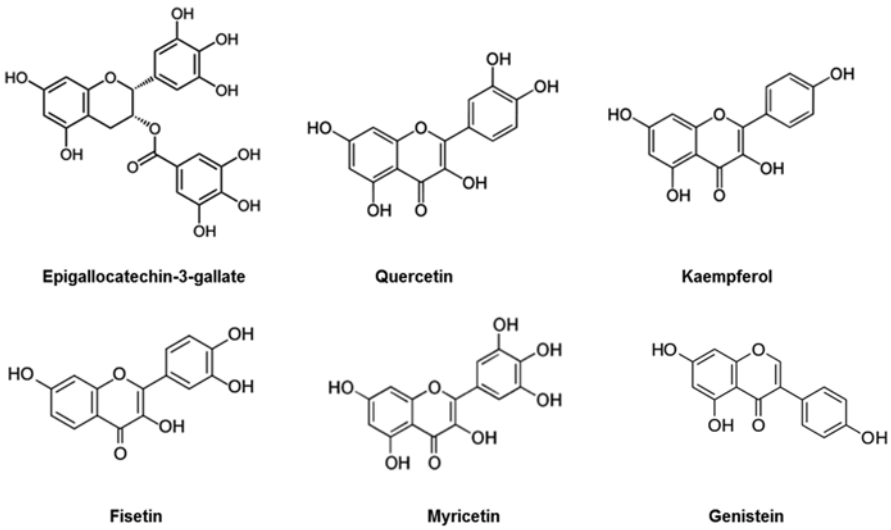


**Fig. 2** Chemical structures of antiangiogenic flavonoids

chemotherapy and induce apoptosis in cancer cells [104, 105]. Antiangiogenic activity of EGCG, demonstrated *in vitro* and *in vivo* [106, 107], could be mediated by inhibition of the VEGF expression, the binding of this growth factor to VEGFR2 or the phosphorylation of this receptor [108–110]. As for tumor cells, EGCG shifts the proteolytic balance of endothelial cells towards anti-proteolysis by downregulation of proteases and upregulation of their natural inhibitors. The antiangiogenic activity of this compound can also be mediated by a direct effect on the activation of HIF-1α and NFκB [111, 112]. Interestingly some data suggest a selective effect of EGCG on tumor-associated endothelial cells and endothelial progenitor cells, responsible for tumor vasculogenesis, but not on normal endothelial cells [113]. In spite of the clear epidemiological evidence regarding the health promoting effect of green tea and the basic research results supporting the EGCG chemopreventive potential, effects observed in clinical trials are not conclusive, probably due to the erratic bioavailability of this compound [114]. Dosage schedules, levels and toxicities derived from potential side effects are some issues that remain to be clarified in further clinical studies [115, 116].

## *4.2 Flavonols*

Quercetin is a flavonol found in many fruits and vegetables, as well as olive oil, red wine, and tea (Fig. 2). It has been well documented that quercetin suppresses tumor growth *in vitro* and *in vivo* by affecting different cell signaling processes usually altered in cancer cells, with limited toxicity on normal cells [117]. While these

studies are promising, clinical trials are needed in order to clearly establish the effects of quercetin on treatment and prevention of cancer. As for many others plant-derived chemopreventive compounds, it has been reported that NFκB may be a target for quercetin [118]. Quercetin inhibits angiogenesis *in vitro* and *in vivo*, affecting several steps of the angiogenesis process, including endothelial proliferation, migration, and tube formation, as well as the expression of MMP-2 [119]. It also inhibits hypoxia-induced VEGF expression in the low micromolar range by a HIF-1 independent mechanism, through suppression of STAT-3 tyrosine phosphorylation [120]. Angiogenesis inhibition by quercetin has been postulated to involve both, suppression of eNOS and early M-phase cell cycle arrest [121], as well as the inhibition of ERK phosphorylation, or the suppression of the VEGFR2 regulated AKT/mTOR/P70S6K signaling pathway in endothelial cells [122]. Quercetin inhibits COX-2-mediated angiogenesis in human endothelial cells in a dose-dependent manner, what suggests a potential use for this compound in the treatment of COX-2-mediated diseases such as breast cancers [123].

The structure of myricetin (Fig. 2) is similar to that of quercetin. This flavonol is found in many plants, including tea, onions, berries, grapes and medicinal herbs. A potent chemopreventive potential for this compound may derive from its antioxidant, antiinflammatory and anticancer effects [124–126]. Myricetin inhibits (UV) B-induced angiogenesis in a mouse skin tumorigenesis model, decreasing the induction of VEGF, MMP-9 and MMP-13 expression. HIF-1 expression and Akt phosphorylation *in vivo* was also inhibited by myricetin, what could be related to a direct inhibition of PI3-K activity [127]. The effect of myricetin on several protein kinases makes it a promising agent for the chemoprevention of skin cancer, as well as other cancer types, by inhibiting both cell growth and angiogenesis [128].

Kaempferol (Fig. 2) is a natural antioxidant, antiinflammatory and antiangiogenic flavonol present in many edible plants, including broccoli, cabbage, tomato, strawberries, grapes and tea, and in plants used in traditional medicine [129]. Kaempferol inhibits angiogenesis and VEGF expression in human ovarian cancer cells through both HIF-dependent (Akt/HIF) and HIF-independent pathways [130]. It downregulates COX-2 expression and the NFκB pathway [131, 132]. Combination of kaempferol and conventional chemotherapeutic drugs increases the antitumor effect and reduces undesired toxicities [133].

Fisetin (Fig. 2), found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber, has been reported as a chemopreventive/chemotherapeutic agent in several types of cancer with neuroprotective and antioxidant properties [134]. It shows antiangiogenic activity *in vitro* and *in vivo*, by inhibition of some key steps of angiogenesis, including endothelial cell growth, survival, migration and tube formation [135, 136]. Fisetin shows a direct inhibitory effect on the activity of several MMPs, what could contribute to the observed inhibition of tumor cell invasiveness and endothelial cell tube formation [135]. Combination of this non-toxic dietary flavonol can markedly improve the *in vivo* antitumor effect of cyclophosphamide with low systemic toxicity, suggesting that this drug combination could advantageously be used in the treatment of solid tumors [137].

### 4.3 Soy Isoflavones

Epidemiological evidence suggests that dietary soy consumption may be beneficial for the prevention or treatment of prostate and breast cancer [138, 139]. Genistein (Fig. 2), originally labeled as a phytoestrogen, is one of the major isoflavones found in soy and has been shown to inhibit cancer growth *in vitro* and *in vivo* [140, 141]. Health benefits of genistein have also been associated to attenuation of postmenopausal problems and a decreased incidence of cardiovascular diseases, among others [142, 143]. Genistein is a multitargeted antitumoral drug, with effect on cell cycle, apoptosis, angiogenesis, invasion, and metastasis. This could be mediated by the inhibition of Akt, NFκB, MMPs and Bax/Bcl-2 signaling pathways [140, 141]. *In vitro*, genistein has been shown to inhibit endothelial cell proliferation at concentrations which are in the range of those found in urine of subjects consuming a plant-based diet. This suggests that genistein could contribute to the cancer preventive effect of a plant-based diet, by inhibiting neovascularization [144].

Genistein downregulates the expression of several molecules responsible for the control of angiogenesis, including VEGF, PDGF, and MMPs, and upregulates angiogenesis inhibitors, such as plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1 [145–147]. The antiangiogenic activity of genistein has been reported to be mediated by the inhibition of HIF-1 in pancreatic carcinoma cells [148] and by the inhibition of PTK activity and MAPK activation in VEGF-stimulated endothelial cell [149].

Results from a Phase 2 clinical trial with patients with localized prostate cancer indicate a possible therapeutic effect by genistein, with no adverse effects of clinical significance. This could explain the epidemiological data indicating a preventive effect of a diet rich in soy products [150]. Nevertheless, further phase 2 trials on bladder, pancreatic and breast cancer showed a lack of biological activity of genistein, making advisable the combined use of this compound with other treatments in order to be clinically effective in the treatment of chemoprevention of cancer [151–153].

### 4.4 Terpenes

Ursolic acid (Fig. 3) is a pentacyclic triterpenoid widely present in edible plants and fruits such as apples, basil, berries, peppermint, rosemary, oregano, and prunes. It exhibits pleiotropic biological effects, some of them probably derived from inhibition of NFκB, what suggests a potential of this compound in cancer chemoprevention [154, 155]. Our results confirmed an inhibitory effect of ursolic acid on different key steps of angiogenesis, including endothelial cell proliferation, migration, differentiation and proteolytic capability [156]. On the other hand, ursolic acid increases the expression of adhesion molecules, angiogenic

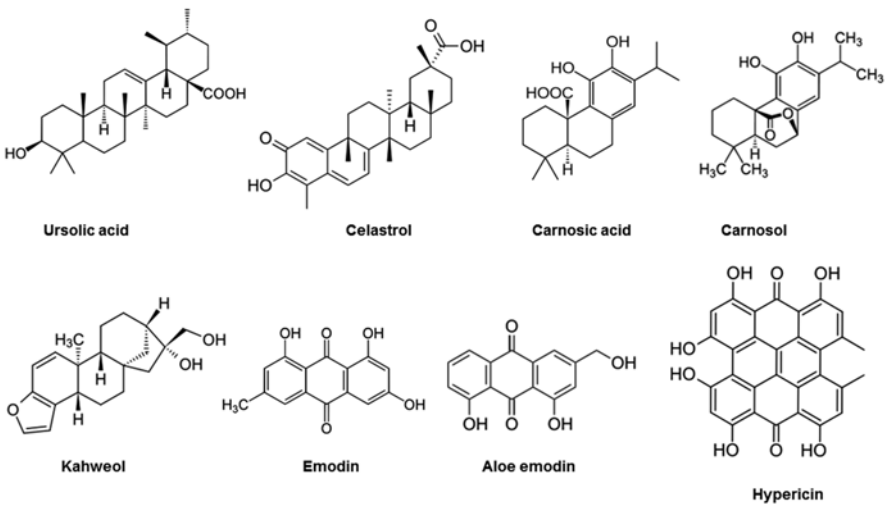


**Fig. 3** Chemical structures of antiangiogenic terpenes, anthraquinones and their derivatives

growth factors such as VEGF and FGF-2 and their receptors, probably mediated through PI3K-Akt pathway [157]. The antitumoral and antiangiogenic potential of ursolic acid has been confirmed in murine models of colorectal cancer and Ehrlich ascites carcinoma tumor without noticeable signs of toxicity [158, 159]. An increasing number of *in vivo* studies indicate that ursolic acid can inhibit tumor initiation, progression, and metastasis in a wide variety of preclinical cancer models. Ursolic acid bioavailability following oral administration has been demonstrated in mice, and clinical data of pharmacokinetic and pharmacodynamics profiles of liposomes containing ursolic acid are also available. Nevertheless, additional clinical trials are required to confirm its efficacy for the prevention and treatment of human cancer [160].

The pentacyclic triterpenoid celastrol (Fig. 3), isolated from the root extracts of *Tripterygium wilfordii* (thunder god vine) exhibits antioxidant, anti-inflammatory and antitumoral properties. Celastrol molecular targets are mostly centered on the inhibition of IκB kinase (IKK)- NFκB signaling [161, 162]. Celastrol have been shown to inhibit angiogenesis through suppression of VEGF receptors and by targeting Akt pathway in endothelial and tumor cells [163, 164]. Celastrol is a potent inhibitor of hypoxia-induced angiogenic and metastatic activity by regulating HIF-1α at multiple levels [165]. The antiangiogenic activity of celastrol seems to be mediated by inhibition of the IKK/NF-κB pathway [166].

The diterpenes carnosol and carnosic acid (Fig. 3), major components of rosemary extracts, have shown activity for cancer prevention and therapy. We have described the antiangiogenic activity *in vitro* and *in vivo* of these compounds, exerted, at least partially, by apoptosis induction on activated endothelial cells, sug-

gesting their potential in the prevention and treatment of cancer and other angiogenesis-related malignancies [167].

Due to its high rates of consumption by humans, coffee can be a major dietary source of chemopreventive compounds. Kahweol (Fig. 3) is an antioxidant diterpene present in coffee beans and unfiltered coffee beverages with high potential pharmacological interest derived from its anti-inflammatory and antitumor activities [168]. We have also demonstrated that kahweol inhibits angiogenesis *in vitro*, *in vivo* and *ex vivo* by interfering several key steps of the angiogenic process. All these data, along with the inhibitory effects of kahweol on endothelial cell COX-2 expression and MCP-1 secretion, clearly indicate that kahweol behaves as a multitargeted antiangiogenic and anti-inflammatory compound with high pharmacological interest [169].

### *4.5 Anthraquinones*

Emodin (Fig. 3), an anthraquinone found in the roots and barks of numerous plants, molds, and lichens, is a tyrosine kinase inhibitor showing antitumor effects [170]. It inhibits endothelial cell proliferation, causing an endothelial cell cycle arrest at G2/M phase, induces endothelial cell apoptosis, and it inhibits MMPs and the tyrosine kinase activity of VEGFR2 [171, 172]. The antiangiogenic activity of emodin has also been related to suppression of the phosphorylation of ERK 1/2 [173]. Recently, a potential antitumor effect of this compound on pancreatic cancer has been proposed, via its dual role in the promotion of apoptosis and suppression of angiogenesis, probably through regulation of NF-κB and Runx2 transcriptional activity [174, 175].

Aloe emodin (Fig. 3) is a hydroxyanthraquinone present in *Aloe vera* leaves, showing antitumor activity [176]. We provided evidence that this compound is also an attractive new inhibitor of angiogenesis, inhibiting the urokinase secretion and tubule formation of endothelial cells, as well as a candidate drug for photodynamic therapy [177]. This has been confirmed by experimental data showing that this compound targets multiple molecules responsible for cellular invasion, migration and angiogenesis, reinforcing the potential of this molecule for the blocking of tumor associated events [178].

Hypericin (Fig. 3) is an anthraquinone derivative found in St. John's wort (*Hypericum perforatum*), a perennial herb that has been widely used as a medicinal plant. We have shown that hypericin kept in the dark inhibits several key steps of the angiogenic process, namely, endothelial cell proliferation, migration, invasion, as well as the extracellular matrix protease production and the formation of tubular-like structures on Matrigel [179]. Hypericin can degrade HIF-1α in cells via a unique hypoxia and proteasome independent mechanism, suggesting that it could be potentially useful in preventing the growth of tumors in which HIF-1α plays pivotal roles [180]. Moreover, hypericin is a powerful photosensitizer used alone or in combination for a selective photodynamic destruction of tumor cells and severe damage of tumor vasculature [181–183].

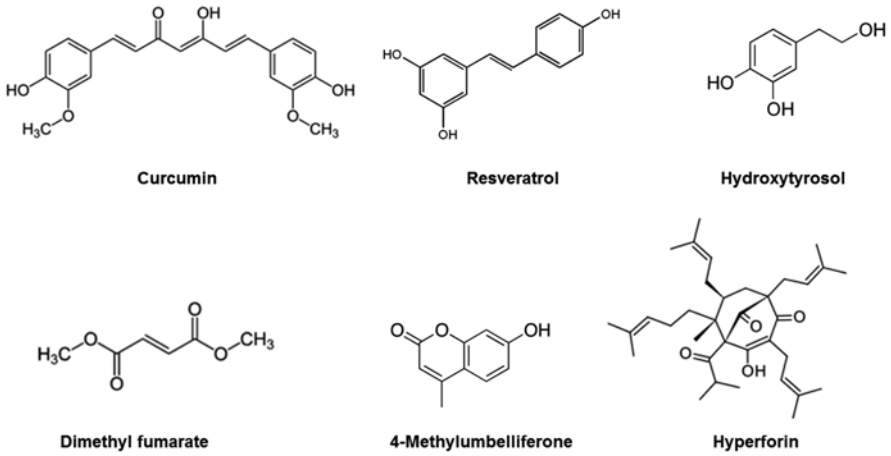


**Fig. 4** Chemical structures of other plant-derived inhibitors of angiogenesis

## *4.6 Other Plant-Derived Angiopreventive Compounds*

The polyphenol curcumin (Fig. 4), an active principle of the perennial herb *Curcuma longa*, has a diverse range of molecular targets, what translates into a widespread effect on diverse biochemical and molecular cascades [184]. Curcumin antitumor activity has been demonstrated in many types of cancer, including oral, lung, breast, prostate, pancreatic, colorectal, multiple myeloma and head and neck squamous cell carcinoma, by means of a number of *in vitro* and *in vivo* studies, clearly revealing its value as a chemopreventive agent [185–187]. It suppresses initiation, progression, and metastasis of a variety of tumors by the downregulation of a number of transcription factors, growth factors, inflammatory cytokines, protein kinases, and other oncogenic molecules (reviewed in [188]). Extensive clinical trials over the past 30 years have addressed the pharmacokinetics, safety, and efficacy of curcumin, either by itself or in combination with other drugs, against cancer in humans (reviewed in [189, 190]). Although the underlying mechanism for the clinical efficacy of curcumin remains unclear, it could be related to the modulation of numerous signaling molecules. Issues such as the poor bioavailability and limited adverse effects should be solved in order to broaden the therapeutic utility of curcumin. Curcumin anti-carcinogenic activity can be related to its effect on the cancer stem cell signaling pathways [191]. The inhibition of angiogenesis by curcumin may be mediated by downregulation of HIF-1, FGF and VEGF and by an inhibitory effect of several signal transduction pathways, including those involving protein kinase C and the transcription factors NFκB and AP-1. The proteolytic capability of endothelial cells is decreased by curcumin, showing an inhibitory effect on two groups of proteinases involved in angiogenesis that are the members of the MMPs and the urokinase plasminogen activator families [192–195]. Interestingly, curcumin can also inhibit alternative mechanisms of tumor vascularization, responsible for some

of the mechanisms of resistance to antiangiogenic therapies. In this regard, it has been shown to suppress the vasculogenic mimicry capacity of hepatocellular carcinoma cells through STAT3 and PI3K/AKT inhibition [196].

Drinking red wine in moderation has long been portrayed as a healthy habit. Resveratrol (Fig. 4), a polyphenolic compound found in grapes and red wine, is an attractive nutraceutical for cancer prevention and treatment [197]. Suppression of the activation of NFκB could render for the tumor prevention potential of this compound [198]. Resveratrol-suppressed cancer growth *in vivo* correlates with the inhibition of angiogenesis [199]. Experimental data suggest that the axis HIF-1α/VEGF could contribute to the angiopreventive activity of this compound [200]. Resveratrol inhibits the VEGF secretion by tumor cells as well as the VEGFR2 activation and expression [200–205]. The observation that resveratrol modulates the expression of VEGF and *in vivo* angiogenesis in a biphasic pattern through the GSK3β/β-catenin/TCF-dependent pathway should be taken into account in the management of cancer and other diseases by the use of this compound [206].

Although in vitro and animal experimental data are extremely promising for resveratrol's anti-proliferative effects, there is limited development regarding its clinical usefulness. A limited number of clinical trials have been carried out to assess the effects of resveratrol on the development and therapy of cancer, and most of them have been focused on colorectal cancer patients. Although clinical data show promising results, additional efforts are required to overcome obstacles such as the limited in vivo bioavailability of this compound and to identify and validate the cellular targets of resveratrol for understanding its health benefits [207, 208].

Hydroxytyrosol (Fig. 4) is a polyphenol found in virgin olive oil, the liquid gold of the Mediterranean diet, showing a number of health-related properties [209]. Results from our laboratory demonstrated for the first time that this compound is a multitargeted inhibitor of angiogenesis [210]. Hydroxytyrosol inhibits MMP and COX-2 expression, as well as the VEGFR2 signaling pathway [209–212]. These findings highlight the properties of olive oil in cancer prevention.

*Fumaria officinalis*, a plant rich in fumaric acid esters, has been in use as treatment for skin complaints since the seventeenth century. Fumarate esters are used primarily in Europe as an oral therapy for psoriasis, with a favourable long-term safety, clinical efficacy profile and relatively low toxicity [213]. The interest of the pharmacologic potential of fumaric acid esters, and mainly that of dimethyl fumarate (DMF) (Fig. 4) has been extended to many other fields of medicine. DMF decreases the expression of proinflammatory mediators by inhibiting the transcription factor NFκB [214]. DMF has been reported to reduce melanoma growth and metastasis in murine models [215, 216], and to enhance the in vivo antitumoral activity of the alkylating agent dacarbazine [217]. Our results clearly established that DMF is a potent inhibitor of angiogenesis, what could help to explain the previously described antipsoriatic, antitumoral and antimetastatic activities of this compound and suggests the interest of this natural product in angioprevention [218].

4-Methylumbelliferone (Fig. 4) is a hydroxycoumarin found in umbelliferous plants, including anise, cumin, parsley, and dill, among others. It is a hyaluronic acid biosynthesis specific inhibitor that has been shown to exhibit antitumoral and

antimetastatic properties, by inhibiting proliferation, adhesion, motility and invasiveness of tumor cells [219–224] Our observations that 4-methylumbilliferone inhibits both *in vitro* and *in vivo* angiogenesis suggest the potential use of this compound in chemopreventive strategies against angiogenesis dependent pathologies, including cancer [225].

Hyperforin (Fig. 4) is a prenylated phloroglucinol derivative that is present in great amounts in St. John's wort, being responsible for most of the antidepressant effects of this medicinal plant. The pharmacological potential of hyperforin also derives from the neurological, inflammation modulatory, antibacterial and antitumoral properties of this compound [226]. Moreover, we have shown that hyperforin behaves as a multitarget antiangiogenic drug by inhibiting endothelial cell growth, invasion, capillary tube formation and the production of endothelial cell MMP-2 and urokinase [227]. We have also shown that hyperforin derivatives with improved antiangiogenic properties can be readily obtained [228]. Hyperforin inhibits nuclear translocation of NFκB, a pivotal transcription factor in the control of both angiogenesis and inflammation, what reinforces the potential of this compound for the chemoprevention of tumour angiogenesis and other inflammatory angiogenesis-associated pathologies [229].

## *4.7 Dietary Proteins and Angioprevention*

Not only small dietary molecules have been related to angiogenesis modulation. Several dietary proteins have been shown to be involved in angiogenesis. Recently, we have reviewed this topic [230]. Herein, we will revise the potential of dietary proteins in angioprevention, with focus on high protein diets, soy proteins and milk proteins.

High protein diets are becoming popular for weight loss. A controlled trial on diets and obesity has revealed that high protein diets with a reduced glycemic index is a good option for weight maintenance [231]. Another randomized controlled trial has shown that a low glycemic index high protein diet, including soluble fiber, inhibits adipogenesis and angiogenesis [232]. These data suggest that high protein diets might exert angiopreventive effects.

Since soy is a main source of proteins for vegetarians, it is advisable to analyze what is currently known on the relationships of soy proteins with angiogenesis. It has been shown that soy protein consumption alters adipocyte transcriptome and metabolome leading to an amelioration of metabolic syndrome in obese rats [233]. Later, the same research group published a study showing that soy proteins can contribute to the maintenance of adipocyte functionality, avoiding adipocyte hypertrophy and impaired angiogenesis [234]. Although this increase of blood vessel area and number in the adipose tissue induced by dietary soy proteins is beneficial in obesity model rats, these data raise concern on the potential pro-angiogenic effects of soy proteins. However, as mentioned before, soy is not only a source of vegetal proteins but also of a number of small bioactive compounds, including the potent

antiangiogenic isoflavone genistein. Therefore, extensive, controlled nutritional trials should be carried out to determine whether vegetarian diets rich in soy have an overall angiopreventive or angioinductive potential.

Milk and its derivatives are essential components of human diets. Several proteins present in milk have been shown to modulate angiogenesis. This is the case of angiogenins, lactadherin, and lactoferrin whose connections with angiogenesis modulation have been reviewed elsewhere [230]. Whereas milk angiogenins 1 and 2 and lactadherin behave as pro-angiogenic compounds [235–237], both lactoferrin and its pepsin-generated derivative lactoferricin exhibit antiangiogenic potential [238]. Therefore, controlled studies should be carried out to determine the overall angiopreventive or angioinductive potential of milk and its dietary derivatives.

## 5 Concluding Remarks

The use of potentially innocuous phytochemicals derived from the diet is an attractive and affordable strategy for the chemoprevention of cancer. Nevertheless, in spite the increasing number of FDA-approved agents for the treatment of precancerous lesions or cancer risk reduction, often cancer chemoprevention trials of dietary agents have achieved a limited clinical success. In recent years, an improved understanding of the molecular basis of carcinogenesis is changing the rationale behind the selection of new strategies for chemoprevention of cancer. Although selection may still be based on historical epidemiological observations, extensive *in vitro* and *in vivo* mechanistic studies, including measures of the effect of the agent on the hallmarks of cancer, are now carried out before clinical evaluation in order to increase the chances to succeed. The common mechanism of action exhibited by many angiopreventive agents suggest the potential identification of a "fingerprint" of the "antiangiogenic switch", shared with other antioxidant, antitumoral or anti-inflammatory chemopreventive compounds. In many cases their effects on several key signaling pathways, such as those of PI3K/AKT and ERK-MAPK, seem to converge on transcription factors such as the nuclear factor NFκB, directly linked to tumor progression.

Identification of surrogate markers and a more logical design of studies of response are needed to support the eventual chemopreventive potentials of these therapies in a timely and cost-efficient manner. This is of special relevance in the case of angiopreventive strategies, since a major clinical challenge in antiangiogenesis is the finding of biological markers that could help to identify subsets of patients more likely to respond to a given antiangiogenic therapy, as well as to determine optimal dosing of therapy, to detect early clinical benefit or emerging resistances and to decide whether to change therapy in second-line treatments. Further studies will be required to develop strategies to optimally exploit the potential of the plethora of available dietary derived inhibitors of angiogenesis to either block the initiation or retard the promotion and progression phases of carcinogenesis. The convergence of the efforts carried out in basic, translational and clinical research

will boost the development of angiopreventive strategies, which will be applied not only to cancer treatment, but also to other diseases characterized by abnormal vasculature for which antiangiogenic approaches have already shown benefits.

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# miRNA as Prognostic and Therapeutic Targets in Tumor of Male Urogenital Tract

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## 1 miRNA Biogenesis and Function

miRNAs are endogenous, single-stranded, short non-coding RNA sequences (about 22 nucleotides) capable to negative modulate the post-transcriptional expression of genes by binding the complementary 3′ untranslated region of mRNA targets. miRNAs work in the translation of targeted mRNA acting as antisense oligodeoxynucleotides. They are synthesized in the nucleus and then transported into cytoplasm where the maturation process is carried out. Furthermore, miRNAs can bind either to the 3′ untranslated region of the mRNA target through imperfect complementarity, or at multiple sites inhibiting the interaction of the mRNA with the ribosomal complex and the translational machinery. Moreover, the not perfect complementarity with the target results in the fact that miRNAs have multiple intracellular targets, and it leads to an amplification of the biological effects. Currently, in the human genome, the number of encoded miRNAs is about 1000. They play an important role in self development, differentiation, proliferation, cell-cycle control, apoptosis and metabolism. Several diseases, such as cancer, have been associated with distinct miRNA signatures, and it means that specific miRNA programs are activated in different pathophysiological processes. Therefore, there has been an exponential growth for the regulatory roles of miRNAs in the development of diseases. Actually, several recent studies indicate that miRNAs could be suitable biomarkers for cancer diagnosis as well as prognostic and therapeutic tools for solid or hematopoietic malignancies [1, 2]. miRNAs, which are upregulated in cancer cells and contribute to carcinogenesis by inhibiting tumor suppressor genes, are considered oncogenic miRNAs (OncomiRs), while downregulated miRNAs, that normally prevent cancer development by inhibiting the expression of proto-oncogenes, are known as tumor

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suppressor miRNAs. The most important advantage in miRNAs is the multiple targeting of different intracellular molecules that results in the amplification of the biological effect induced by miRNA. Similarly to the treatment of tumours with target based agents, the targeting of multiple signal transduction components can be useful in overcoming the redundancy of tumorigenic pathways in cancer cells. It is also of crucial importance to avoid the so-called off-target effects induced by miRNAs in normal tissues and thus it becomes essential to deliver the nucleic acids specifically in tumour tissues sparing normal counterparts [3].

## 2 Testicular Cancer

Testicular cancer accounts for only 1 % of all cancers in men. In the United States, about 8000 men are diagnosed with testicular cancer, and about 390 men die of this disease each year [4]. Testicular cancer occurs more often in men between 25 and 40 years old, and is the most common form of cancer in men between 15 and 34 [5, 6]. The incidence and mortality linked to testicular cancer vary according to geographical distribution. In Northern Europe (7.8 %), Western Europe (6.7 %), Australia (6.5 %), and North America (5.1 %), the incidence rates are highest, while the South Europe (4.2 %) and Central America (3.7 %) have a lower incidence, and the lowest rates are observed in Asia and Africa (1 %). Testicular cancer mortality is highest in Central America (0.7 %), Central-Eastern Europe (0.6 %) and Western Asia (0.6 %), the lowest mortality rates were found in Australia (0.1 %) and Eastern Asia (0.1 %) [7].

The exact causes of testicular cancer are not known. However, studies have shown that several factors could increase a man's chance of developing this disease [8–14]:

- undescended testicle (cryptorchidism): the risk of testicular cancer is increased in males with a testicle that does not move down into the scrotum. This risk does not change even after surgery to move the testicle into the scrotum and the increased risk concerns both testicles;
- congenital abnormalities: men born with abnormalities of the testicles, penis, or kidneys, as well as those with inguinal hernia may be at increased risk;
- family history of testicular cancer: the risk for testicular cancer is greater in men whose brother or father has had the disease;
- Klinefelter's syndrome: different malignancies such as breast cancer, testicular tumors, has been associated with this syndrome;
- presence of contralateral tumor: the prevalence of contralateral tumor accords well with the known prevalence of bilateral testicular tumours;
- infertility: there is an increased risk of testicular cancer in infertile men.

More than 90 % of cancers of the testis develop in special cells known as germ cells. Based on the characteristics of the cells in the tumor, testicular cancers are classified as **seminomas** or **nonseminomas**. Seminomas may be one of three types:

*classic*, *anaplastic*, or *spermatocytic*. Types of nonseminomas include *choriocarcinoma*, *embryonal carcinoma*, *teratoma*, and *yolk sac tumors*. Many testicular cancers contain both seminoma and non-seminoma cells. These mixed germ cell tumors are treated as non-seminomas because they grow and spread like non-seminomas [15, 16]. Testicular germ cell tumor (TGCT) initially is a non-invasive form of the disease called carcinoma in situ (CIS) or intratubular germ cell neoplasia. In testicular CIS, the cells look abnormal under the microscope, though they did not spread outside the walls of the seminiferous tubules. It is hard to find CIS before the developing in invasive cancer because it generally does not cause symptoms; the only way to diagnose testicular CIS is the biopsy [17, 18]. Tumors can also develop in the supportive and hormone-producing tissues, or stroma, of the testicles. These tumors are known as gonadal stromal tumors. They make up less than 5 % of adult testicular tumors but up to 20 % of childhood testicular tumors. The two main types are *Leydig cell tumors* and *Sertoli cell tumors*. These tumors are often benign and usually do not spread beyond the testicle and may be cured with surgery. But in case of metastasis in other tissues or organs, usually they become resistant to conventional chemotherapy or radiotherapy [15, 16].

## 3 miRNAs as Diagnostic and Prognostic Biomarkers in Testicular Cancer

For testicular germ cell tumor prognosis, classical biomarkers α-fetoprotein (AFP), β-human chorionic gonadotropin (βHCG) and lactate dehydrogenase (LDH) are elevated in only 60 % of all patients. Therefore, new diagnostic and prognostic biomarkers are warranted. Several studies have shown that the expression profile of miRNAs can currently provide information for cancer prognosis. Recent studies have shown that miRNAs are aberrantly expressed in human tumors and have been identified in all types of biological fluids, including cerebrospinal, pleural and peritoneal fluid, urine, tears and saliva; therefore, they represent a novel class of useful biomarkers in cancer.

Voorhoeve P. M. et al. examined a number of cell lines originating from TGCTs for the expression of the miR-371-3 cluster; they showed that miR-372 and miR-373 are up-regulated and strictly localized to the stem cell component. Moreover, they found miR-372 and-3 to collaborate with RAS and stimulate a neoplastic transformation phenotype in the presence of wild type p53 with direct target of LATS2 (Large Tumor Suppressor Homolog 2), this suggests that the suppression of LATS explains the sustained activity of CDK in the presence of high p21 levels in miR-372/3-expressing cells [19–21]. McIver S.C. et al. have shown that miRNAs play a key role in spermatogenesis and in the development of testicular cancer [22]. Since small changes in the expression of miRNAs could be significant in tumorigenesis, McIver SC et al. examined, in mice, the expression profile of miRNAs during the stages of development, particularly in the transition between postnatal gonocytes

and spermatogonia, which is crucial in the development of TGCTs. They identified seven differences in the expression of miRNAs; miR-136, -743 and -463* were over-expressed, while miR-290-5p, -291a-5p, -294* and -293 were under-expressed [23]. The analysis of their targets indicated that these miRNAs affect PTEN and Wnt/β catenin pathways and CXCR4 signaling pathway [24]. PTEN tumor suppressor, through the inhibition of PI3K signaling causes a negative regulation of cell growth. Its loss is associated with increased proliferation and tumorigenesis [25]. The loss of another negative regulator of PKI, PIK3IP1, increases the rate of relapse in TGCT [26]. Both PTEN and Wnt/β catenin pathways control the function of Cyclin D1, which is expressed in highly proliferative TGCTs and is associated with resistance to chemotherapy in these tumours. On this basis, the control of this Cyclin can be considered as a possible target [27]. To determine the targets of miR-291a-5p, -293 and -743 it was used a knockdown assay by which has been identified as a possible target the Insulin-like growth factor binding protein 7 (IGFBP7) which is known to act depending on the environment in which it is found, for example as either a positive (e.g., in glioma) or negative regulator (e.g., in breast and liver cancer) of cell proliferation and migration [23]. Gillis et al. in their studies show that miR-155 are over-expressed in seminomas and type III germ cell tumours compared to normal testicles. Furthermore, they found in the same tumors, an over-expression of miR-19a and 29a while miR-133a, -145 and -146 were under-expressed [28]. Syring I et al. explored the role of serum miRNAs as novel biomarkers in patients with testicular germ cell tumor. They observed a high specificity between testicular germ cell tumor and the presence of miR-367-3p and 371a-3p in serum. In particular, miR-367-3p, 371a-3p, 372-3p and 373-3p were significantly increased in patients with cancer compared to healthy individuals and patients with non-malignant testicular disease. Serum miRNA levels were increased in patients with advanced local stage and metastases; in nine patients with localized (clinical stage 1A) testicular germ cell tumor, serum miR-371a-3p levels decreased post-operatively, and indicating tumor specific release. In addition, they found high sensitivity (84.7 %) and specificity (99.0 %) of serum miRNA levels that could be useful in identifying testicular germ cell tumor well as surveillance monitor therapy and residual disease after chemotherapy. In this way, the analysis of miRNAs provides better results than the classical tumor markers AFP and HCG that increase of only 19.8 % and 40.7 %, respectively, in patients with malignant testicular cancer. This method could be very useful to distinguish between seminoma and non seminoma since the seminoma patients often are markers negative [29]. Recent studies examined the global miRNA expression in CIS cells, in particular were analyzed miRNAs expressed in these cells by identifying those whose expression is correlated with the ratio of tubules with CIS cells. In accordance with similar expression profiles of mRNA, it was found a strong correlation between miRNA in CIS cells and gonocytes. In embryonic stem cells, testicular and extragonadal paediatric germ cell tumours only two miRNA clusters, has-miR-371-373 and -302-367, are expressed. These miRNAs were also detected in CIS cells and in faetal gonads, which would leave thinking, that CIS cells are arrested gonocytes in the adult testis and do not differentiate into spermatogonia. In accordance with this, miRNAs that

**Table 1** Regulation of mi-RNAs in testicular and bladder cancer

| mi-RNA | Regulation | Reference |
|---|---|---|
| Testicular cancer | | |
| miR-371-3 cluster | Up | Voorhoeve et al. [19] |
| miR-136, -743, 463* | Up | McIver et al. [23] |
| miR-290-5p, -291°-5p, -294*, -293 | Down | McIver et al. [23] |
| miR-21, -155, miR-19a, -29a | Up | Gillis et al. [28] |
| miR-133a, -145, -146 | Down | Gillis et al. [28] |
| miR-367-3p, -371a-3p, -372/3-3p | Up | Syring et al. [29] |
| hsa-miR-9, -105, -182,-183,-96 | Up | Novotny et al. [30] |
| has-miR-515-526 cluster | Up | Novotny et al. [30] |
| Bladder cancer | | |
| miR29a/b/c, -7, -30, miR-99-101, -125, -129, -145, -195 | Down | Zabolotneva et al. [31] |
| miR-10, -21, -23a/b, -103, -221, -223 | Up | Zabolotneva et al. [31] |
| miR-452, -222 | Up | Puerta-Gil et al. [32], Zhang et al. [33] |
| miR-143 | Down | Puerta-Gil et al. [32] |
| miR-99a, miR-125b | Down | Zhang et al. [34] |
| miR-409-3p | Down | Xu et al. [35] |
| miR-19 | Up | Feng et al. [36] |
| miR-200c, miR-141, miR-30b | Down | Ratert et al. [37], Mahdavinezhad et al. [38] |
| miR-214 | Up | Kim et al. [39] |
| miR-145 | Down | Dyrskjot et al. [40] |
| miR-21 | Up | Dyrskjot et al. [40] |
| miR-30-3p, miR-133a, miR-199a* | Down | Ichimi et al. [41] |
| miR-96, miR-183 | Up | Yamada et al. [42] |
| miR-200, miR-155, miR-192 | Down | Wang et al. [43] |

are differentially expressed between CIS cells and foetal gonads were also identified: hsa-miR-9, -105 and -182-183-96 clusters were highly expressed in seminoma, while hsa-miR-515-526 cluster was high in embryonal carcinoma, indicating that the developmental arrest of gonocytes leading to CIS cells can be imposed by miRNAs. Therefore, miRNA expression profile changes during testis development and miRNA profile of adult testis with CIS cells shares characteristic similarities with the expression in foetal gonocytes [30]. For a summary see Table 1.

## 4 Bladder Cancer

Bladder cancer is the seventh most common cancer among men with estimated 386,300 new cases and 150,200 deaths in 2008 worldwide [44]. Bladder cancer mostly occurs in males and the incidence varies according to geographical

distribution. In Europe, North America and Northern Africa the incidence rates are highest; in particular, Egyptians males have a mortality rate higher in absolute (16.3 per 100,000) than males in Europe (8.3 in Spain and 8.0 in Poland) and Americans (United States 3.7) while Melanesia and Middle Africa have a lower incidence. In Western countries, cigarette smoking and occupational exposures are the main risk factors, while in developing countries such as Africa and Middle East the chronic infection with Schistosoma hematobium encumbers for about 50 % [45]. It is estimated that over 20 % of cancers worldwide can be linked to infectious agents. In this regard, *Schistosoma haematobium* is a blood parasite that resides in venules and capillaries of the human bladder; it is endemic in Africa and Middle East [46]. Indeed, in the Egyptian population, studies confirm that clinical history of urinary schistosomiasis is associated with increased bladder cancer risk, explaining about 16 % of bladder cancer cases [47]. The molecular mechanisms underlying this association are still unclear. Probably schistosomiasis triggers an irritation and inflammation of the bladder tissue, with consequent release of free radicals which lead to the formation of carcinogenic metabolites in addition of being responsible of gene mutations [48–51]. Through a control of schistosomiasis, attendance rates and incidence of bladder cancer have significantly fallen. However, despite the strong decrease of bladder cancer associated with *Schistosoma*, this disease is still the most common cancer among males in Egypt, probably for an increase of bladder cancer due to tobacco consumption. The two main histological types of bladder cancer are transitional cell carcinoma (TCC), which represents more than 90 % of malignant tumors of the urinary tract and not transitional cell carcinoma (non-TCC), 5–7 % of all bladder cancers. The latter is rare and its origin is not completely clear; therefore, the clinical treatment is still under discussion. Moreover, it has a different biological behavior as compared to TCC, with about 3 % of squamous cell carcinomas (SCC) that arise through a process of squamous metaplasia; 2 % of adenocarcinomas (ACs) that is more frequent in males in their sixth decade of life; 1 % of undifferentiated carcinomas (UCs) and a lower percentage of minor histologies as small-cell carcinomas and lymphomas [52–55]. AC of the urinary bladder has an aggressive biological behavior, and is mostly muscle-infiltrating at diagnosis; it is male predominant with the mean age of 62 years and 72.3 % of patients are smokers. According to its origin it can be classified into primary, urachal and metastatic and histologically classified as enteric, signet-ring cell, mucinous, clear cell, hepatoid, mixed or AC not otherwise specified (NOS). The tumor type non-TCC is generally a very aggressive cancer with low survival rates; it is recommended an initial radical cystectomy to improve the prognosis.

The likelihood of developing this disease appears to increase with age, the probability of having bladder cancer at age 39 is about 0.02 % while at age of 70 years the possibility of developing the disease rises to 3. 69 %. At diagnosis, 75 % of cases of bladder cancer are located in the mucosa and submucosa, while 25 % are tumors that have already infiltrated the muscles [37]. The symptoms of bladder cancer that include discomfort during urination, hematuria, and urgency of urination unfortunately are nonspecific and may be common with other diseases such as the urinary tract infections. Therefore, an accurate and clear diagnosis can be obtained by

examining the cells under a microscope both from urine and bladder tissue or cystoscopy. However, these procedures are invasive and sometimes require the use of anesthesia, especially when it needs a biopsy. Non-invasive methods, such as the control of cells in the urine, often are not as effective because they depend on the stage of the disease [56]. Ideal biomarkers to detect a disease should first be inexpensive, noninvasively detected, specific for the disease and detectable before clinical symptoms. miRNAs may be useful as non invasive prognostic markers in the case of urothelial carcinoma; urine is a particularly desirable source of these biomarkers and their levels in the urine rarely change; moreover, miRNAs exist in a stable form in the urine even after several cycles of freezing [57]. In bladder cancer when histologically matched with normal urothelium, various types of mi-RNAs are aberrantly expressed.

## 5 miRNA as Diagnostic and Prognostic Biomarkers in Bladder Cancer

Several studies have recently shown that miRNAs are emerging as a new class of cancer biomarkers. The identification of the expression profile of miRNAs in cancer cells and the comparison with those of healthy cells, could be a valuable tool in the development of the diagnosis and prognosis of bladder cancer. Zabolotneva et al. identified 95 miRNAs that were differentially expressed in bladder cancer tissues and seven miRNAs that were differentially methylated in bladder cancer vs. non-cancer patients. They used subtractive suppression hybridization (SSH), and (DS) technologies to determine miRNA expression profiles in 17 tissue samples of urothelial bladder carcinoma and in eight histologically normal urothelial samples. In particular, they found that miR29a/b/c, -7, -30, miR99-101, -125, -129, -143, -145, and -195 were down-regulated while miR-10, -21, -23a/b, -103, -221 and 223 were up-regulated [31]. Tumor-suppressive and oncogenic roles of miR-221 and miR-222 have been reported, suggesting they have a bimodal function in the tumorigenesis of human cancers. Oncogenic roles of miR-221 and miR-222 have been shown in several types of human malignancies, especially in breast cancer. In this light, (locked nucleic acid-inhibitors) (LNA-i-) miR-221 exerted strong antagonistic activity against miR-221 and induced upregulation of the endogenous target p27Kip1 in multiple myeloma [58–60]. These miRNAs exert their oncogenic abilities through suppressing the cyclin-dependent kinase inhibitors p27Kip1, and p57 or upregulating the ZEB2 gene through TRPS1 [61–65]. Interestingly, miR-29 family is considered an epi-miRNA gene family having broad regulating role on DNA methyltransferases and histone deacetylases (HDAC) that, in turn, have a pivotal role in the epigenetic regulation of myeloma [66–68]. It was found that miR-452 is involved in tumorigenesis as well as in the diagnosis of bladder cancer, whereas miR-143 and miR-222 may be related to tumor progression and used for the evaluation of clinical results. Differential expression of miR-143, miR-222, and miR-452 in cells present in urine were verified by *in situ* hybridization in matching

tumors. Cancer cells displayed low levels of expression for miR-143 and higher levels of expression for miR-222 and miR-452. Moreover, miRNA expression by RT-qPCR was correlated with tumor grade, size, and presence of carcinoma *in situ* for miR-222, recurrence (miR-222 and miR-143), progression (miR-222 and miR-143), disease-free survival (miR-222), and overall survival (miR-222). Protein expression profiles of targets regulated by these miRNAs include VEGF, Bcl2, erythroblastic leukemia viral oncogene (ERBB3) homolog 3, and ERBB4 [32]. Furthermore, Zhang DQ and colleagues found that miR-222 might be involved in both carcinogenesis and metastasis of bladder cancer; in fact, the expression level of miR-222 was significantly associated with tumor stage and grade, indicating that high miR-222 levels are a marker of poor prognosis for patients with bladder cancer. The authors of this study confirmed the clinical importance of miR-222 expression by showing its association with unfavourable clinic-pathological features and poor survival. The molecular mechanisms of miR-222 seems to be related to PI3K/AKT pathway, which is considered to play a major role in bladder carcinogenesis [33, 69]. Bladder cancer progression was associated with two major signaling pathways: (1) abnormal activation of the fibroblast growth factor receptor 3 gene (FGFR3) and (2) aberration of p53 pathway. The first signaling pathway participates in the activation of the RAS-kinase that leads to increased cell proliferation, motility, and cancer transformation through hyperplasia of normal urothelium. The alteration of the second pathway leads to the development of carcinoma *in situ*, invasive carcinoma, and metastases through urothelial dysplasia [70–72]. In this context miR145, miR143, miR101, miR7, miR99a, and miR100 targeted the FGFR3 gene product; in particular, an increase in miR143 expression is paralleled by the lower expression of RAS gene in bladder cancer cells; a decrease in miR7 expression is frequently associated with hyperactive FGFR3 mutation status in bladder cancer tissues [73]. miR10, miR129, targeted MDM2, MDM4, and ATM gene products, while miR125b, miR30a/c, miR223 were predicted to target p53 directly [74]. Recent studies [64] have shown that the supernatant of the urine of patients with big hematuria, was more homogeneous in the analysis of the profile of microRNAs compared to total urine probably because it eliminates the interference due to the cellular contamination. Specifically, in patients with urinary carcinoma, miRNAs profile was determined and compared with those of healthy control patients by microarrays analysis of the supernatant of urines. The analysis revealed the presence of over 100 miRNAs that are most under-regulated than upregulated in cancer patients' urine supernatants. In details, miR-99a and miR-125b are down-regulated in a manner proportional to the grade of the tumor. MiRNA-99a and miRNA-125b can be, therefore, used as effective biomarkers detection and disease monitoring of urothelial carcinoma of the bladder. They can also function as therapeutic tools for the treatment of these patients. A high expression of phosphorylated mTOR was found in 74 % of muscle-invasive urothelial carcinomas, in association with increased pathological stages and decreased disease-related survival. Moreover, the inhibition of mTOR was found to decrease *in vitro* and *in vivo* bladder cancer cell growth. Based on these findings, it is likely that miR-99a targets mTOR also in bladder cancer as already reported in prostate cancer cells [75]. In bladder cancer, miR-125b inhibited both

colony formation in an *in vitro* cell model and *in vivo* tumor development in nude mice by targeting E2F3, which frequently showed to be overexpressed in bladder cancer and had an expression inversely correlated with that one of miR-125b. Moreover, a diagnostic model was developed using a combined index of the levels of miR-99a and miR-125b in the urine supernatant with a sensitivity of 86.7 %, a specificity of 81.1 % and a positive predicted value (PPV) of 91.8 %. Discriminating between high- and low-grade of urothelial carcinoma of the bladder, the model using the level of miR-125b alone exhibited a sensitivity of 81.4 %, a specificity of 87.0 % and a PPV of 93.4 % [34]. Finally, miR-125b was recently reported to target IRF4 in multiple myeloma cells, suggesting an important role as anti-cancer agent [76]. Another study shows that the expression level of miR-409-3p is significantly lower in the cells and tissues of the bladder tumor compared to healthy cells and tissues. Mirna-409-3p is able to suppress migration and invasion, and may serve as a metastasis-suppressor gene. By using the real-time PCR, western blot and luciferase assays, it was found that miR-409-3p exerts its effect by targeting c-Met. Precisely miR-409-3p plays a role in the suppression of metastasis in bladder cancers by downregulating, at least partially, the protein expression of c-Met. Therefore, it may be possible to develop miR-409-3p as a novel therapeutic strategy for bladder cancer therapy [35]. The deregulation of miR-19 has been identified in several types of cancer, in particular, it is associated with the tumor growth and metastasis; therefore, miR-19 has been identified as the key member responsible for the oncogenic activity [77–79]. Feng Y and colleagues found that the levels of miR-19a in bladder cancer tissues are upregulated and the high expression of miR-19a has been associated with more aggressive phenotype of bladder cancer. Moreover, the authors show that miR-19a plays its oncogenic role in bladder cancer in part by targeting PTEN. Finally, they studied the expression of miR-19a in the plasma of bladder cancer patients. It was increased also in the plasma of the patients thus suggesting that miR-19a could be considered as a potential diagnostic marker of bladder cancer [36]. Several studies have been performed on miRNA profiling of bladder cancer concluding that miR-200c and miR-141 are upregulated in bladder cancer patients compared to healthy control group suggesting that they can be used for both diagnosis and clinical outcome prediction [37, 80–82]. Heejeong Lee et al. [81] studied the expression of four miRNAs (miR-145, miR-205, miR-125b, and miR-200c), which have been reported to be involved most frequently in bladder cancer. They found that the expression of miRNA-145, miRNA-205, miRNA-125b, and miRNA-200c is significantly lower in high grade papillary urothelial carcinoma (HG) than in low grade papillary urothelial carcinoma (LG). In addition, the authors show that zinc finger Ebox binding homeobox 1 (ZEB1) and zinc finger Ebox binding homeobox 2 (ZEB2) are direct target genes of these miRNAs. Mahdavinezhad and colleagues have evaluated the expression levels of miR-30b, miR-141 and miR-200c in tissue samples of patients with bladder cancer; their results indicated that the values were significantly higher than those of the control samples of normal tissue. miR-141 had a higher rate of expression in malignant tissues if compared to the other two miRNAs [38]. When the comparison is made between the invasive and non-invasive, some studies show that expression of miR-30b, miR-141 and miR-200c was reduced

in invasive bladder cancer. Therefore, low levels of these three miRNAs indicate an invasive type and a poor prognosis [83]. As a consequence, miR-200c, miR-141 and miR-30b can be potentially used to diagnose invasive bladder cancers that were misdiagnosed in pathologic assessment of bladder biopsy specimens [84]. Cell-free miRNAs in urine could be direct indicators of urological conditions including injury and malignancy. Kim SM and coll. measured the expression levels of miR-214 in the urine of non-muscle-invasive bladder cancer patients. They have found that these patients had relatively high levels of miR-214 compared with the control patients. The authors speculate that miR-214 may be associated with non-invasive disease due to its effect of tumor suppression through inhibition of angiogenesis and cell proliferation. These results indicated that urinary miR-214 might serve as a non-invasive biomarker for predicting the prognosis of bladder cancer [39]. Dyrskjot et al. investigated the expression of 290 human miRNAs in 106 bladder cancer samples and in 11 normal samples using spotted locked nucleic acid-based oligonucleotide microarrays. Their study demonstrated that miR-145 was the most down-regulated in cancer compared with normal, while miR-21 was the most up-regulated in cancer. In addition, they have identified a correlation between miRNAs and presence of concomitant carcinoma *in situ*. In particular by *in situ* hybridization, they localized the expression of miR-145, miR-21 and miR-129 in the urothelium. Moreover, they documented a direct link between miR-129 and the two putative targets GALNT1 and SOX4 [40]. In another study, researchers identified miRNAs that have a tumor suppressive function in bladder cancer, in particular they found a subset of 7 miRNAs (miR-145, miR-30a-3p, miR-133a, miR-133b, miR-195, miR-125b and miR-199a*) that were significantly downregulated in bladder cancer differently from those of normal epithelium. Furthermore, their target search revealed significant inverse correlations between Keratin7 mRNA expression and each down-regulated miRNAs. Interestingly, Keratin7 mRNA was significantly reduced by transfection of a bladder cancer cell line with the three miRNAs (miR-30-3p, miR-133a and miR-199a*). In addition, significant decrease of cell growth was observed after transfection of three miRNAs and siRNA-Keratin7 in the same cells, suggesting that miR-30-3p, miR-133a and miR-199a* may have a tumor suppressive function through the mechanism underlying repression of Keratin7. These miRNAs can be used as promising candidates for biomarkers and gene therapy of human bladder cancer [41]. It was also recently reported that miR-199 is involved in the regulation of neo-angiogenesis in human cancers disclosing a new function for miR-199 in the control of human cancer microenvironment [85]. Using miRNA microarray profiling and qRT-PCR, it was investigated the expression levels of miRNAs in urine samples from 104 urothelial carcinoma patients and evaluated the correlations of their expression and clinicopathological features. The expression levels of miR-96 and miR-183 in the urine samples were significantly higher in 100 urothelial carcinoma patients than in healthy controls. The gradual increase of these miRNAs was correlated with both advanced tumor grade and pathological stage. In addition, the expression levels of these microRNAs were significantly lower in urine collected after surgery suggesting that miR-96 and miR-183 in urine could be promising tumor markers for urothelial carcinoma. The gene expression profile of miR-96 and

miR-183 transfectants demonstrated that the downregulated gene categories seem to include tumor suppressive categories, for example, anatomical structure development, regulation of signal transduction, cell differentiation and apoptosis. BCL2-associated X protein (BAX) was the top of the common downregulated genes in both miR-96 and miR-183 transfected cells. These results implied that miR-96 and miR-183 are onco-miRNAs and might be a potential target for gene therapy of some human malignancies [42]. The epithelial-to-mesenchymal transition (EMT) process that occurs during embryonic development but which plays a key role in the metastatic progression of bladder cancer, is controlled by members of the miR-200 family. In fact, members of the miR-200 family appear to control the EMT process and sensitivity to EGFR therapy in some of the bladder cancer cells. ZEB1, ZEB2, and ERRFI-1 are three examples of miR-200 direct targets; in fact, their down-regulation by miR-200 is associated with an EGFR-sensitive phenotype. These results should help us to prospectively identify bladder tumors that will be most susceptible to EGFR-directed therapy [86, 87]. In another study, authors analyzed by qRT-PCR the expression of miRNAs in both urinary sediment and cell-free urinary supernatant of bladder cancer and non-cancer patients. They found that the expression of miR-200 family members, miR-155, and miR-192 was down-regulated in the urine sediment of patients with bladder cancer and that these levels were increased after removal of the tumor tissue which suggests that bladder cancer is the direct cause of the depressed urinary miRNA levels. The expression of the miR-200 family, miR-205, and miR-192 in the urine sediment significantly correlated with urinary expression of EMT markers, including zinc finger E-box-binding homeobox 1, vimentin, transforming growth factor β1, and Ras homolog gene family, member A. It is probably that down-regulation of miR-200 family facilitates EMT process promoting cancer progression. These findings suggest that miR-200 family plays an important role in the pathogenesis of bladder cancer, and may represent a non-invasive, diagnostic and prognostic biomarker for bladder cancer [43]. For a summary see Table 1.

## 6 Prostate Cancer

Prostate cancer (PCa) is the most common solid tumor in men in developed countries, and the second cause of death for cancer, after lung cancer. It is estimated that PCa affects about 2.8 million men in the USA; it hits older men and the incidence increases with age. In fact, the median age at diagnosis is 67 years. The percentage probability of developing pCa increases with age: 0.01 % for those under 40 years of age, 2.63 % for those aged between 40 and 59 years, 6.84 % for those between 60 and 69, and 12.54 % for those aged over 70 years. In industrialized countries, PCa incidence and mortality are up to 20-fold higher than developing countries; nutrition and other lifestyle factors have been suggested to be a cause for this difference. Diet and exercise altered serum factors that slowed the growth rate and induced apoptosis in androgen-dependent PCa cells. In contrast, high levels of body mass index (BMI), blood pressure, and a composite score of all metabolic factors were associated with

increased risk of PCa death. Numerous data demonstrate significant ethnic differences in mortality rates; Caribbean have the highest rates in the world (26.3 per 100,000), followed by Sub-Saharan Africans (10 per 100,000), while Asians have the lowest (2.5 per 100,000). This ethnic variation is likely due to genetic and environmental factors. In addition, there is a difference in the risk of PCa among men of different populations in the United States, in particular African Americans have the highest risk of developing PCa. Basically, the diagnosis of PCa is based on three different parameters: (1) digital rectal exam (DRE), (2) serum concentration of PSA and (3) transrectal ultrasound guided biopsy. In about 18 % of all patients, a suspect DRE alone detects PCa; furthermore, a suspect DRE in patients who have a PSA level up to 2 ng/ml has a positive predictive value of 5–30 %. However, even if a higher level of PSA indicates the presence of a PCa, it has been established that some patients develop this cancer despite low PSA levels [3]. Conversely, a high serum PSA level may be consequence of some other factors such as a benign prostatic hyperplasia, an inflammation of the gland or some drugs [88]. The diagnosis of PCA basically depends on histopathologic or cytologic confirmation, especially in the so-called grey zone, namely in patients with PSA 4–10 ng/ml. Consequently, there is often an over-diagnosis and sometimes an over-therapy with patients undergoing surgical or pharmacological therapy without an effective need. Therefore, it is strongly warranted the finding of new biomarkers both for diagnosis and for prognosis of prostate cancer, avoiding in this way invasive tests such as biopsy or DRE.

## 7 miRNAs as Diagnostic and Prognostic Biomarkers in Prostate Cancer

The challenge is finding specific miRNAs that could be able to distinguish between normal and cancer cells. Unfortunately, to date there are still some controversial results concerning the expression of some miRNAs in prostate cancer. In 2006, Volinia et al. [89] showed that miR-21, miR-17-5p, miR-191, miR-29b-2, miR-199a-1, miR-146, miR-181b-1, miR-20a, miR-32, miR-92-2, miR-214, miR-30c, miR-25 and miR106a were all up-regulated in prostate cancer, whereas Porkka et al. showed a down-regulation of 37 miRNAs and an up-regulation for 14 miRNA [90]. A recent study demonstrated that miR-224 inhibits invasion and migration of PCa cells by targeting apelin, a target gene of miR-224. Suppressing the expression of apelin, this miRNA inhibits cell invasion and migration; furthermore, a down-regulation of miR-224 was significantly associated with advanced clinical stage and metastasis [91]. miR-21 is one of the most commonly deregulated oncomiR in cancer; in PCa, miR-21 expression increases together with clinical parameters (such as Gleason score or lymph node metastasis) and is correlated with castration resistance and metastatic disease [88]. Therefore, miR-21 is also useful as a biomarker in prediction of progression of cancer. Also miR-143 and miR-145 are inversely correlated with Gleason score. As for prognostic markers, in bone metastasis, Peng et al. demonstrated that miR-508-5p, miR143, miR-145, miR33a, miR-100 were inversely

correlated with metastasis, so that their expressions decreased in case of metastatic disease in prostate cancer. Though the role of miR-141 in bone metastasis is not well known, its expression is positively correlated with expression of bone alkaline phosphatase, that is an important marker for bone metastasis [92]. Also, the role of miR-141 in prostate cancer is still unclear, but it has been shown that it is up-regulated after castration, so that it is possible that it plays a role in androgen mechanism which is both important in androgen-dependent and in metastatic castration-resistant PCa. It demonstrates the importance of miR-141 in studying cancer progression. However, the most interesting result concerning the role of miRNAs in cancer is their expression in body fluids such as serum, for a new and non invasive tool for diagnosis: circulating miRNAs could be an answer for risk, staging and even prognosis in cancer. Mitchell et al. showed for the first time in 2008 that tumor related miRNAs can be found in body fluids and so easily measured and that miR-141 was higher in the serum of patients affected by PCa and this result was subsequently confirmed in various studies. The importance of this result is that miR-141 could be an important biomarker for PCa diagnosis and prognosis for the future. In fact, it has been shown that some miRNAs could distinguish between low vs. intermediate vs. high risk patients with PCa [90]. One of these studies found that 28 miRNAs were up-regulated and 30 miRNAs were down-regulated in a group of patients with unfavorable vs. favorable prognosis [93]. Another important study about circulating miRNAs demonstrated that besides miR-141, also miR-375 showed a deregulation in non metastatic patients and a relevant importance for an early diagnosis [94]. It was also found an association between miR-34 family members and PCa tissue with ability in differentiating neoplastic from tumour tissue [95]. In fact, miR-34 family was widely reported as a tumour suppressor in cancer and several miR-34-replacing strategies have been developed to treat cancer [96–100]. One of the most important factors limiting therapy based upon miRNAs is the systemic delivery of miRNAs or antago-miRNAs in tumor tissues. On this light, we have recently demonstrated that the use of stealth liposomes or of new nanoparticles based upon calcium phosphate and cationic liposomes could be efficiently used for the delivery of anti-cancer agents in prostate cancer tissues [101, 102]. Therefore, it is possible to imagine the co-encapsulation of miRNAs with other active anti-cancer agents for the treatment of prostate cancer.

For a summary see Table 2.

## 8 Renal Cancer

Kidney is an important organ and its function consists in balancing electrolytes, filtering the blood and wasting ammonium compounds; several studies described the presence of miRNAs both in normal kidney and in various diseases, including cancer. Renal cell carcinoma (RCC) is the most common type of renal cancer, though the definition includes various types of cancer occurring in the kidney, and each subtype seems to be characterized by different chromosomal alterations [99].

**Table 2** Regulation of mi-RNAs in prostate and renal cancer

| mi-RNA | Regulation | Reference |
|---|---|---|
| Prostate cancer | | |
| miR-21, miR-17-5p, miR-191, miR-29b-2, miR199a, miR-146, miR181b-1, miR-20a, miR-32, miR92-2, miR-214, miR-30c, miR-25, miR106-a | Up | Volinia et al. [89] |
| miR-224 | Down | Wan et al. [91] |
| miR-21 | Up | Cannistraci et al. [88] |
| miR-143, miR-145, miR508-5p, miR-143, miR-145, miR-33a, miR-100 | Down | Zhang et al. [92] |
| miR-141 | Up | Kim et al. [39] |
| Renal cancer | | |
| miR-197, miR-215, miR-217, miR-155, miR-1826, miR-143, miR-26a, miR145, miR-10b, miR-195, miR-126, miR187 | Down | Li et al. [103] |
| miR-506 | Down | Yang et al. [104] |

Furthermore, RCC may be divided into five subtypes with clear cell renal cell carcinoma (ccRCC), the most common type. The other ones are: papillary RCC (pRCC), the chromophobic RCC (chRCC) and oncocytomas (the rarest). This tumor heterogeneity and the absence of pain make diagnosis difficult, so that in general there is a late presentation. Renal cell carcinoma is the eighth cancer in men, and unfortunately one third of the patients present metastasis at the diagnosis because there are no screening test for this disease. Another 50 % of the patients develop metastasis during the therapy: in fact, RCC has only 10 % of survival after 5 years, because in general there is no responsiveness to both chemotherapy and radiotherapy [105].

## 9 miRNAs as Diagnostic and Prognostic Biomarkers in Renal Cancer

It has been widely studied that miRNAs play an important role in tumorigenesis and metastasis, so that it is crucial to find new biomarkers for a non invasive and early diagnosis. It has been shown that miR-141 and miR-200c are down-regulated in RCC, with up regulation of their common target ZFHX 1B leading to the attenuation of CDH 1/E-cadherin transcription. Furthermore, the levels of the following miRNAs and their targets: miR-143 (Hexokinase-2), miR-145 (ADAM 17, ANGPT2, NEDD9), miR-10b, miR-195, miR-126 decrease in RCC relapse patients, and in particular two of them (miR-145 and miR-126) seem to indicate low survival in non metastatic RCC so that they may be considered as prognostic factors [106]. Recently, interesting results concerned a study about miR-497, which is a tumor

suppressor in various cancers: it was significantly decreased in RCC tissues, compared with normal tissues. Moreover, the miRNA levels were strictly related to a higher tumor stage and to the presence of lymph node or visceral metastases, while Kaplan-Meier survival analysis showed that the survival rate in patients was shorter when the expression of miR-497 decreased compared to patients with a higher expression of the miRNA. All these results confirm the importance of this biomarker both in diagnosis and prognosis, and that it could be also useful in the choice of therapy. Moreover, *in vitro* experiments showed that miR-497 inhibited proliferation, migration and invasion capability of cancer cells; it could be a potential prognostic marker and function as tumor suppressor in human cervical cancer by post-transcriptionally targeting IGF-1R [107]. As for body fluid miRNAs, Redova et al. found 19 miRNAs upregulated and 11 miRNAs downregulated in RCC patients; patients with metastases showed higher circulating levels of miR-221 than patients without metastases. In details, miR-141 is able to distinguish between ccRCC tissues and normal one; in fact, it is a suppressor gene for ccRCC cell proliferation and metastasis by modulating the EphA2/p-FAK/p-AKT7MMPs signaling cascade, and then it can distinguish oncocytoma from RCC and ccRCC from oncocytoma. As for prognostic factors, the downregulation of miRNAs strictly correlated to poor prognosis (in terms of survival or recurrence) include miR-187, miR-215, miR-217, miR-155, miR-1826, miR-143, miR-26a, miR-145, miR-10b, miR-195, and miR-126. They all presented a lower expression in tumor tissues. These prognostic biomarkers are of crucial importance because unfortunately metastases are quite common in RCC and some of these miRNAs (-10b, -139, -5p, -130b, -199b-5p) are associated with ccRCC metastasis. The circulating miRNA expression reveals that miR-187 decreased in patients with higher stage: patients with a high expression survived at least 5 years [103]. Another interesting work showed that miR-506 was downregulated in ccRCC, and in particular that a lower expression was associated with a poor prognosis and an advanced stage. An overexpression of miR-506 decreased cell growth and metastases in renal cancer cells, while a downregulation increased them. The target gene, FLOT1, was inversely correlated with miR-506 expression: a knockdown by siRNA inhibited malignant cells [104]. For a summary see Table 2.

## 10 Conclusions

A growing amount of data is suggesting that intratumour miRNAs can be useful in order to distinguish normal and cancer tissues and that it can be predictable of the clinical outcome of the patients. Moreover, miRNAs can be also efficiently and easily assessed with low cost and rapid technologies in the body fluids of the patients (i.e.: plasma, urine etc.) and that they can be again correlated with the prognosis and the sensitivity to therapy of human cancers. The appearance of miRNAs on the dawn line of cancer research has opened an intriguing new scenario in which the assessment of the diagnosis and prognosis of cancer patients can be performed with

a simple and easy-to-do blood or urine collection. However, a huge amount of clinical data obtained on large series of patients are requested in order to clarify the role of circulating miRNAs in human cancers including the male urogenital tract cancers. The design of large multi-centric studies collecting a significant amount of normal and cancer tissues, plasma and urinary samples from cancer patients are suggested in order to obtain conclusive data on miRNAs. Moreover, the development of easy and rapid kits for the detection of circulating miRNAs using nanotechnological devices are also strongly warranted in order to allow the diffusion of these markers in the health care systems.

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