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Analysis of the MicroRNAs MiR-34a/b/c as Mediators of the Effects of Curcumin and Aspirin on Colorectal Cancer

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I hereby declare, that the submitted thesis entitled:

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Table of content

List of abbreviations

CRC	Colorectal cancer
PTGS1	Cyclooxygenase 1
PTGS2	Cyclooxygenase 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
PGE2	Prostaglandin E2
5-FU	5-Fluorouracil
DOX	Doxycycline
EMT	Epithelial-to-Mesenchymal Transition
MSI	Microsatellite instability
qCHIP	Quantitative chromatin immunoprecipitation
NRF2	Nuclear factor-erythroid factor 2-related factor 2
ROS	Reactive oxygen species
KEAP1	Kelch-like ECH-associated protein 1
AMPK	AMP-activated protein kinase
NQO1	NAD(P)H: quinone oxidoreductase 1
TBHP	tert-Butyl hydroperoxide
USPSTF	United States Preventive Services Taskforce
EZH2	Enhancer of zeste homolog 2
HMGB1	High-Mobility Group Box 1
MRX34	Liposomal formulation of miR-34a
COX	Cyclooxygenase
TNF	Tumor necrosis factor

List of publications

1. Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis

Chunfeng Liu, Matjaz Rokavec, Zekai Huang and Heiko Hermeking. Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis. Cell Death and Differentiation, 2023.30(7): p.1771-1785.

2. Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cascade resulting in suppression of colorectal cancer metastasis

Chunfeng Liu, Matjaz Rokavec, Zekai Huang and Heiko Hermeking. Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cascade resulting in suppression of colorectal cancer metastasis. Cell Death and Disease. 2023 Oct 28;14(10)*:* 707.

Your contribution to the publications

1.1 Contribution to paper I: Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis

As the first author of this paper, the doctoral candidate searched literatures and had close consultation with her doctoral supervisor Pro. Heiko Hermeking and postdoc Matjaz Rokavec, who jointly conceived this program. Under the supervision of her doctoral supervisor Prof. Heiko Hermeking, the doctoral candidate independently conducted experiments and analyzed the results. The doctoral candidate prepared a draft of the manuscript, including creating all tables, figures, and accompanying materials (Supplementary Material). The text, figures, and tables of the manuscript were repeatedly revised by her doctoral supervisor Prof. Heiko Hermeking, and then the doctoral student together with postdoc Matjaz Rokavec revised the manuscript based on her doctoral supervisor's comments.

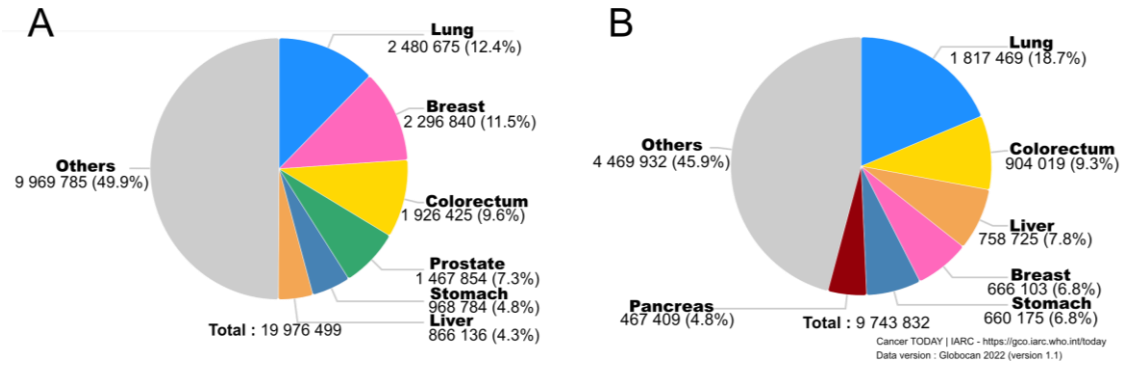
1.2 Contribution to paper II: Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cascade resulting in suppression of colorectal cancer metastasis

As the first author of this article, the doctoral candidate reviewed literatures and repeatedly discussed with her doctoral supervisor Prof. Heiko Hermeking and postdoc Matjaz Rokavec, who jointly conceived the research. Under the supervision of her doctoral supervisor Prof. Heiko Hermeking, the doctoral candidate independently conducted experiments and analyzed the results. The doctoral candidate prepared a draft of the manuscript, including creating all tables, figures, and accompanying materials (Supplementary Material). Throughout the process, she consulted with doctoral supervisor Prof. Heiko Hermeking and received his guidance and feedback. She got advice and support every step of the way from her doctoral supervisor Prof. Heiko Hermeking.

2. Introduction

2.1 Colorectal cancer

Colorectal cancer (CRC) develops in the colon (the large intestine) or rectum (the end of the large intestine). CRC ranks as the 3rd most common type of cancer worldwide, with nearly 2 million new diagnoses reported in 2022. It is the 2nd most common cause of cancer-related death, accounting for nearly 1 million deaths annually (Figure 1.1).



A. Number of new cases in 2022 B. Number of new deaths in 2022

Figure 1.1 Global Cancer Statistics 2022**. (A)** Estimated number of new cancer cases worldwide in 2022, for both sexes and all ages. **(B)** Estimates of cancer deaths worldwide in 2022 for both sexes and all ages related to cancer. Source: GLOBOCAN 2022, https://gco.iarc.fr/today/en/dataviz/pie?mode=population&group_populations=0.

CRC has a complex genetic and environmental etiology. Multiple risk factors have been linked with the development of CRC, including MSI (microsatellite instability), polygenic mutations, and disease. However, about 75% of CRCs are sporadic, occurring without genetic predisposition or a family history of CRC [1]. The incidence of CRC varies widely between countries. For example, Northern and Western Europe, North America, and New Zealand have the highest rates of incidence, while South Central Asia has the lowest rates of incidence (Figure 1.2) [2]. Therefore, the role of diet and lifestyle in the occurrence of CRC has received attention recently. A large amount of epidemiological evidence shows that environmental factors (such as diet and lifestyle) are closely related to the development and progression of CRC [3-5]. One study showed dietary factors accounted for almost 50% of all colorectal cancer diagnoses, whereas the family history-attributable risk was only about 10% [6]. Therefore, a healthy diet and lifestyle, or additional interventions to modify these risk factors, are considered for the primary prevention of CRC.

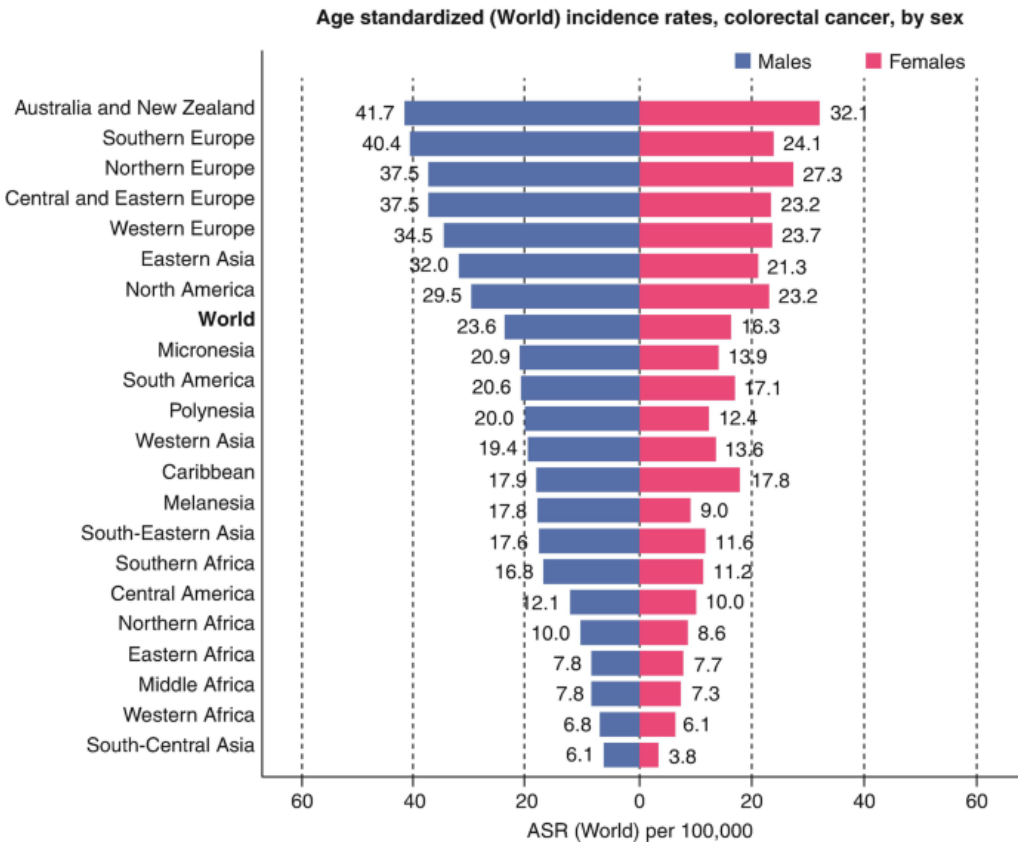


Figure 1.2 CRC incidence in men and women in different regions of the world [7].

CRC is a multi-step, multi-stage, and multi-gene cytogenetic disease [8]. Vogelstein and Fearon [9] proposed an adenoma-carcinoma sequential model of the development of CRC, according to which adenomas may need decades to develop into cancers and ultimately distant metastases (Figure 1.3A). The model divides CRC into four distinct stages: early adenoma, late adenoma, carcinoma, and metastatic carcinoma. Since the development of CRC is a long-term process, there is an opportunity to effectively prevent colon cancer. Sporn et al. [10] first proposed chemoprevention in 1976, which refers to using natural or synthetic substances to prevent and delay the occurrence of cancer, or reverse the cancer process, thereby preventing tumor recurrence and metastasis (Figure 1.3B). An ideal chemo-preventive drug usually has good tolerance, few side effects, and is easy to administer. Also, it should effectively prevent cancer occurrence and development, reverse the cancer process, and prevent tumors from returning and spreading.

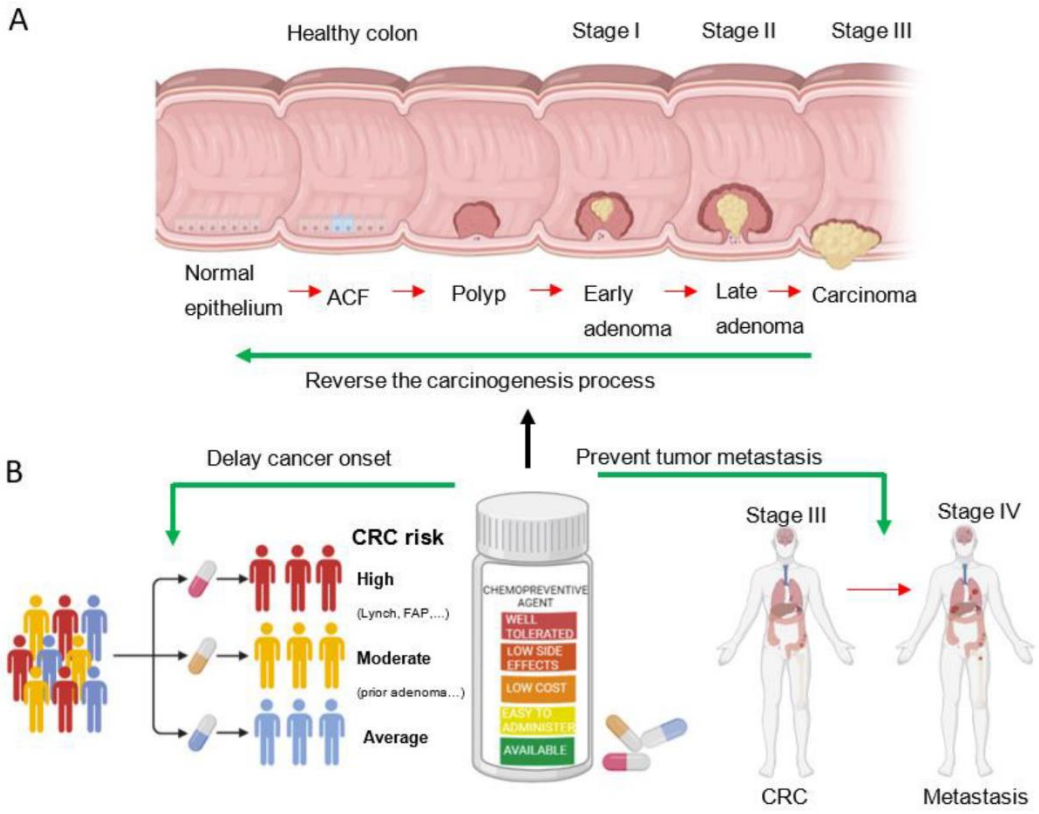


Figure1.3 (**A**). adenoma-carcinoma sequential model of CRC development. (**B**). Chemo-preventive agents are natural and synthetic compounds aimed at delaying cancer onset, reversing the carcinogenesis process, and preventing tumor recurrence and metastasis [11].

Surgery and chemotherapy are currently the main treatments for CRC. Chemotherapeutics kill cancer cells by causing DNA damage or activating various signalling cascades and cellular processes (such as cell cycle arrest, DNA repair, etc.). Cytotoxicity, drug resistance, nausea, vomiting, pain, and other side-effects are common chemotherapy side effects. Through the study of compounds from plants, animals, and microorganisms, many anti-cancer drugs have been discovered. Alternatively, natural ingredients could be good candidates for chemo-preventive or chemo-therapeutic agents for CRC patients to prolong living. Many studies reported that natural chemicals prevent colorectal cancer growth by modulating multiple signalling pathways and processes, such as apoptosis, proliferation, invasion, metastasis, autophagy, and angiogenesis (Figure 1.4).

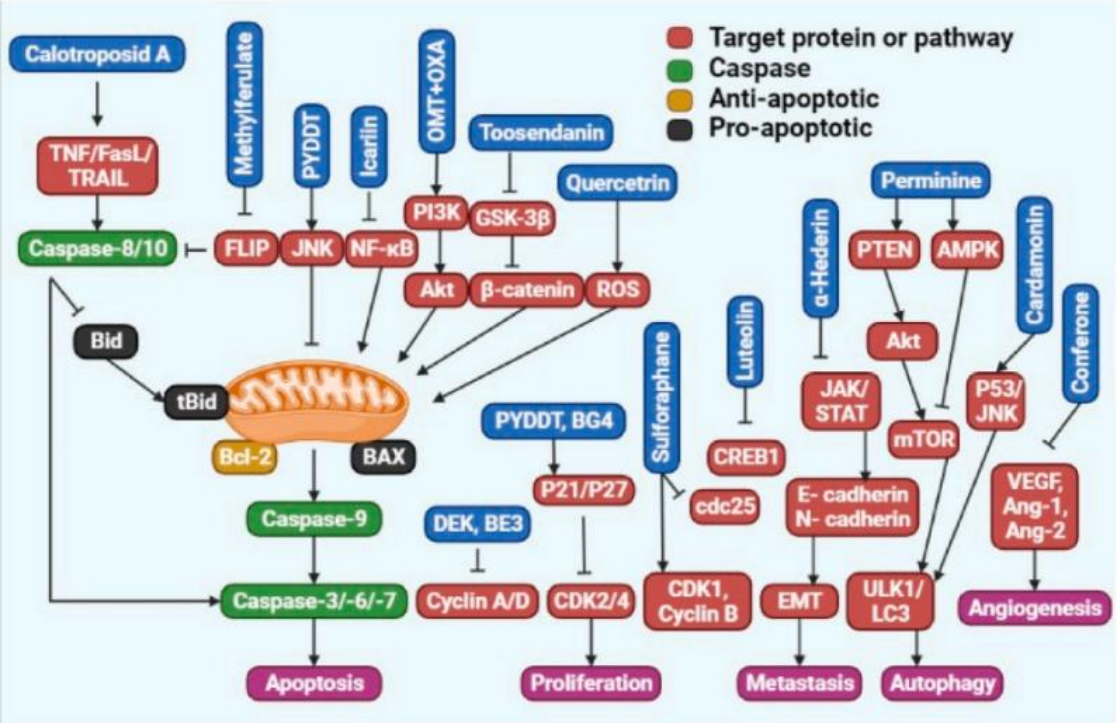


Figure1.4 Molecular mechanisms and regulatory networks of natural chemicals in colorectal cancer [12].

Phenolic chemicals appear to be the main active molecules behind these anticancer effects. Now polyphenols have been extracted and identified from many plants. Polyphenols in green tea have been shown to protect against multiple chemically induced malignancies and suppress the growth of cancer cells and tumor development in cancer models in vitro and in vivo. Curcumin, a naturally occurring polyphenol with pleiotropic pharmacological characteristics including anti-oxidant, anti-inflammatory, and anti-cancer potential, is considered one of the safest alternatives for treating CRC [13]. Curcumin has molecular effects on IGF [14], VEGF [15], IL-1 [16], COX-2 [17], IL-6 [18], and chemokines in CRC. For example, curcumin represses the growth and induces apoptosis of CRC cells via regulating the Akt/mTOR signal pathway to repress the expression of EGFR [15]. Curcumin not only regulates tumor suppressors and transcription factors but also has the ability to regulate miRNAs through epigenetic mechanisms, which include changes in DNA methylation [19] and histone modifications [20]. Such as curcumin decreases the expression of *miR-21* by reducing the activity of the *miR-21* promoter and suppressing the binding of AP-1 to the *miR-21* promoter, thereby repressing the proliferation and metastasis of tumor cells [21]. Curcumin could significantly inhibit the mRNA level of *EZH2* (enhancer of zeste homolog 2), which is a histone methyltransferase enhancer, to increase the levels of the *let-7* family [22] and *miR-200* family [23], thereby repressing the proliferation and metastasis of tumor cells.

Acetylsalicylic acid is currently the most extensively studied NSAIDs (nonsteroidal anti-inflammatory drugs) and is considered to be one of the most promising chemo-preventive agents for colorectal cancer. Studies have found that about 40% of colonic adenomas and 90% of sporadic CRC have increased expression of COX-2 [24,25]. Additionally, the high expression of PGE2 in colon cancer has been shown to be a predisposing factor for CRC [26]. A well-known effect of aspirin is the irreversible binding, acetylation, and subsequent inhibition of cyclooxygenase 1 (PTGS1) and cyclooxygenase 2 (PTGS2), causing the down-regulation of prostaglandin E2 (PGE2) [27]. Beginning in 1982, multiple trials showed that an effect of aspirin in chemo-prevention for colorectal adenomas [28-31]. Taking low dose aspirin daily for 5 to 10 years can decrease the rate of cancer mortality by about 20% [32].

Non-aspirin NSAIDs (NA-NSAIDs) also have been reported to have chemo-preventive effects on CRC. Takayama et al [33] found that the size and number of colonic polyps were decreased in FAP patients after treatment with sulindac (300 mg per day). A study of patients taking celecoxib (200 mg or 400 mg, two times every day) found a dose-dependent reduction in adenoma incidence of 33 - 45% after 3 and 5 years [34,35]. In patients diagnosed with colorectal adenomas, a daily intake of 25 mg of rofecoxib reduced adenoma formation by 24% [36].

Aspirin exerts overall health benefits in the chemoprevention of CRC. The USPSTF (United States Preventive Services Taskforce) has ample proof that taking low-dose aspirin decreases the incidence of CRC in adult after application for 5 to 10 years. Therefore, the USPSTF recommends that adults of 50 to 59 years should be initiated on low-dose aspirin as primary prevention of CRC.

2.2 The role of p53 in CRC

2.2.1 The role of p53 in CRC

Genomic and epigenetic alterations are common in colorectal cancer, leading to gene inactivation and subsequent promotion of tumor formation, and represent the driving force of tumorigenesis. Mutations in the *p53, APC*, and *K-Ras* genes are known to be associated with CRC progression [37]. In human cancers, *p53* is the most frequently mutated gene [38] and plays a critical role in the adenoma-carcinoma transition in tumor pathology [39]. *P53* mutations were present in 45% of distal colon tumors and 34% of proximal colon tumors [40,41]. Since *p53* mutations lead to accumulation of p53 protein, Rodrigues et al. [42] found that approximately 50% of primary colorectal cancers overexpressed p53 protein, while benign adenomas did not display p53 overexpression.

2.2.2 Effects of p53 in CRC

In healthy or unstressed cells, p53 levels remains low due to negative regulation by MDM2 and its chaperone MDM4 (MDMX) [43]. In response to cellular stress or DNA damage, p53 accumulates and co-ordinates multiple responses by activating multiple transcriptional targets, including components of metabolic, anti-oxidative, and anti-angiogenic processes, causing cell cycle arrest, autophagy, and apoptosis, thus preventing tumor progression and development (Figure 1.5) [44,45].

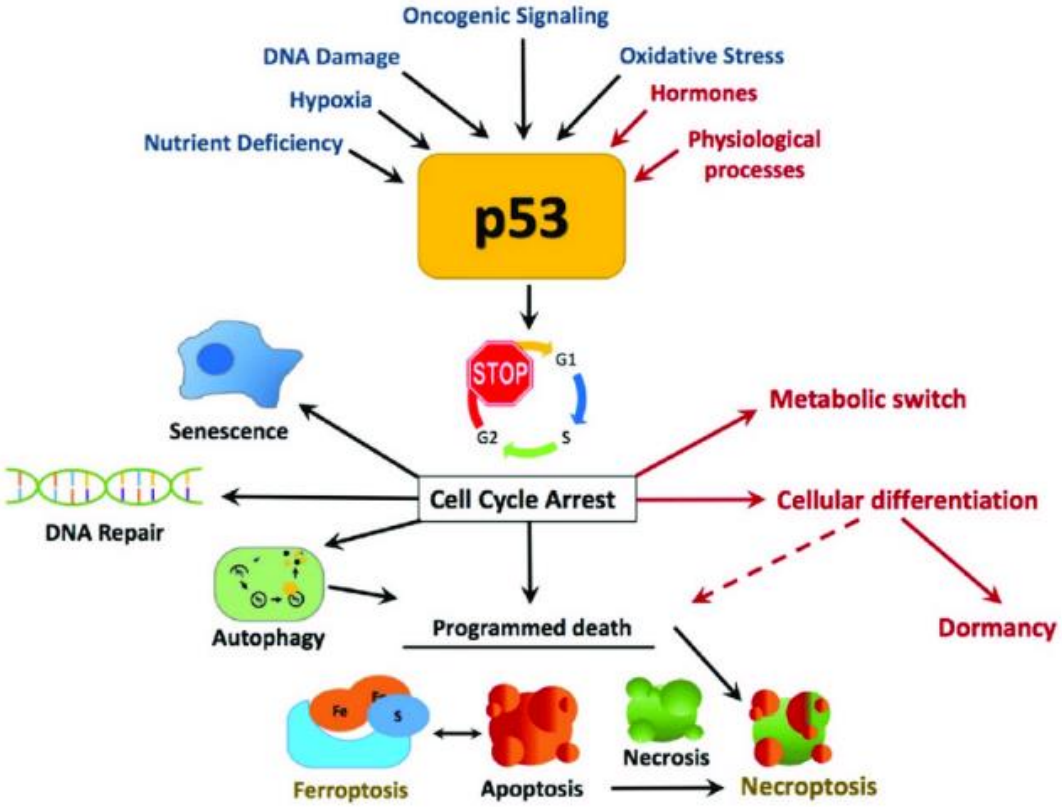


Figure 1.5 The function of p53. Picture taken from [46].

The tumor suppressor function of p53 is mainly exerted by inducing tumor suppressive genes. After DNA damage, p53 activates DNA repair genes, allowing the cell to survive. If the mutated DNA can not be repaired, p53 induces apoptosis of cells [47,48]. P53 induces apoptosis via activating Bcl-2, BAX, PUMA, and Noxa in CRC cells. Furthermore, p53 also regulates the activity of cell death receptors such as Fas or DR5 and induces apoptosis by activating the caspase signaling pathway [49]. In addition, p53 also regulates the activity of p16, p21, and PML to induce cellular senescence [50]. P53 may maintain the stability of the genome by reducing the levels of reactive oxygen species (ROS) [50-52]. In addition to its direct effect on cells, p53 can also prevent tumor progression by affecting the tumor microenvironment [53].

2.2.3 Mutant p53 in CRC

More than half of CRC have a *p53* gene mutation [54], which plays a key role in the transition from adenoma to cancer [55,56]. Missense mutations replacing GC with AT (48%) are the most

frequent *p53* mutations in CRC [57,58]. Mutations mostly cluster in the DNA-binding domain of p53, leading to the disruption of the binding of p53 to its target genes and their transactivation [59]. In tumor tissues, mutated p53 proteins accumulate by evading MDM2 degradation [60-62].

P53 mutations have been shown to determine the biological behavior of CRC, for example, metastatic site, depth of invasion, and patient prognosis [63]. *P53* mutations are linked with lymphatic invasion in proximal CRC and are significantly associated with lymphatic and vascular invasion in distal CRC [40]. Thus, in patients with metastatic CRC, the *p53* mutation rate is 80% [64]. Conversely, *p53* mutations are uncommon in benign colorectal adenomatous polyps, with a frequency of 15% to 30% [65]. Mutation of *p53* leads to widespread activation of NF-κB and Wnt/β-catenin signal pathways, promotes the maintenance and regulation of the undifferentiated state of tumor cells, and enhances invasion, EMT, and metastasis in CRC [55].

In late-stage CRC, loss of WT *p53* is also found beside missense mutations. *P53* deficiency promotes VEGF secretion and neovascularization, thereby promoting tumor progression [66]. The deletion of wild-type *p53* activated EMT-like processes in colorectal cancer, proliferation, and resistance to therapy [67-69]. Therefore, loss of *p53* is more likely to be observed in moderately or poorly differentiated tumors and is linked to metastasis of lymph nodes [70]. Ramona et al. [71] found that targeted deletion of *p53* in mouse intestinal epithelial cells, while not sufficient to induce CRC, significantly increased the aggressiveness and incidence of tumors following treatment with the carcinogen azoxymethane.

In addition to regulating protein-coding genes, p53 also acts tumor-suppressive by inducing or inhibiting the expression of certain miRNAs [72]. Through the regulation of miRNAs, p53 influences biological processes, such as cell proliferation, apoptosis, invasion, and metastasis [73,74]. miRNAs are important mediators of p53 for tumor suppressor function. In addition, miRNAs can positively regulate p53 expression by directly inhibiting negative regulators of p53, such as SIRT1 and MDM4 [75,76].

2.3 The p53/miR-34 connection

In 2007, several research groups, including the Hermeking lab at the MPI of Biochemistry, reported that among all miRNAs, miR-34a and miR-34b/c were detected to be most highly induced by p53 [77-82], and their hosting genes were confirmed as p53-target genes. Since then, numerous studies have shown that *miR-34a* family members represent important mediators of p53-induced inhibition of tumor initiation and progression (Figure 1.6) [83-86].

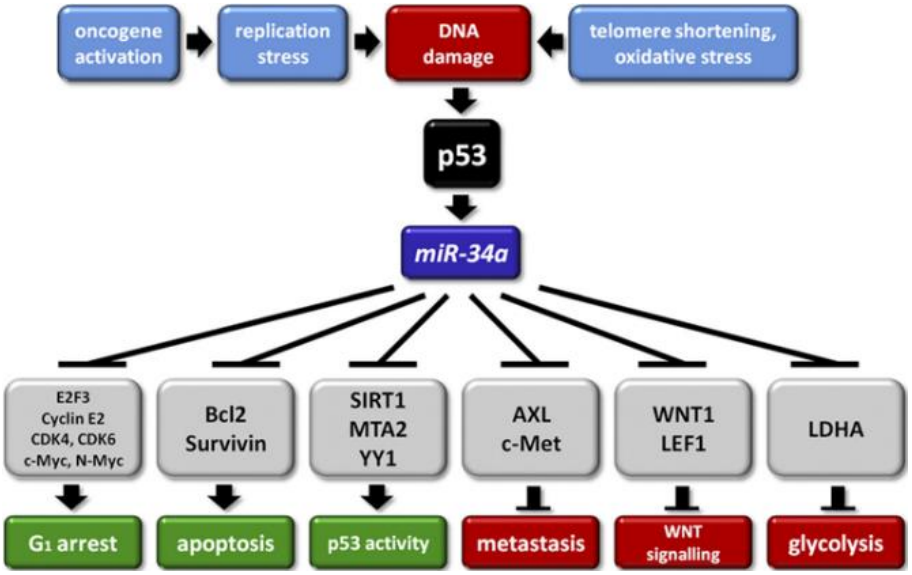


Figure1.6 The effect of the p53-miR-34a axis on multiple biological processes/pathways. Taken from [87].

Surprisingly, deficiency of the *miR-34a* and/or *miR-34b/c* genes alone in mice does not appear to advance the rate of tumor formation [88]. However, *miR-34a/b/c* deficiency resulted in an increased tumor burden and decreased survival in the *Apc*^{Min} mouse model of intestinal cancer [89]. Furthermore, *miR-34a* deficiency facilitated tumor invasion in a mouse model of colitis-associated cancer [90]. Combined inactivation of *miR-34a* and *p53* leads to an increase of tumors, promoting the invasion and lymph node metastasis of surrounding tissue in the 6xAOM mouse model of CRC [91].

Although miR-34a levels are lower in *p53*-deficient or *p53*-mutant cells, it is still expressed, suggesting the existence of a p53-independent regulatory mechanism for *miR-34a* expression. [88,92]. A p53-independent mechanism of *miR-34a* regulation was reported to involve the transcription factor ELK1, and AXL, and JNK pathways [93]. On the other hand, E2F1 [94], SMAD4 [95], and c-MYC [96] have been shown to repress *miR-34a* expression. For example, B-RAF induced the expression of *miR-34a* via targeting the proto-oncogene *c-MYC* [93]. Certain stress stimuli, such as oxidative stress, DNA damage, and oncogenic signaling, were reported to induce *miR-34a* expression through p53-independent pathways [97,98]. In summary, *miR-34a* is regulated by both, p53-dependent and p53-independent mechanisms. The effect of *miR-34a* on cell cycle and apoptosis is independent of p53. It has been shown that ectopic miR-34 induced apoptosis and cell viability [99] as well as p21 expression (a downstream gene of p53) in *p53* +/+ and *p53* −/− cells [97].

2.4 miR-34

2.4.1 Inactivation of *miR-34* genes in cancer

MiRs (miRNAs) are a type of small endogenous non-coding RNAs that can specifically bind to the 3'-non-coding region of messenger RNAs (mRNAs), suppressing mRNA translation or inducing mRNA degradation. The first miRNA (*Lin-4*) was discovered in 1993 by Victor Ambros and colleagues while studying defects in the timing control of development in Caenorhabditis elegans [100]. Ambros et al. discovered that the *Lin-4* gene does not encode a protein, but instead produces a pair of small RNAs of about 22 and 61 nucleotides in length (the longer one is thought to be the precursor of the shorter one), which interacts with *Lin-14* [100,101]. The miRNAs bind to partially complementary sites in the 3' untranslated region (UTR) of the mRNA to control developmental timing in C. elegans. The shorter *Lin-4* RNA is thought to be the first member of a still-growing family of miRNAs. Subsequent studies have shown that the link between miRNA dysregulation and human disease is relevant in almost all medical fields. Furthermore, miRNAs also regulate physiological processes, such as embryonic, fat metabolism, cell proliferation and cell death. Numerous studies showed that miRNA expression profiles are altered in tumors [102-104], suggesting that they play a critical role in the development and progression of cancers. Furthermore, miRNAs are also important for cancer diagnosis and prognosis.

MiR-34 is a member of an evolutionarily conserved miRNA family originally discovered in Caenorhabditis elegans and has three members (miR-34a, miR-34b, and miR-34c) [105]. In mammals, the miR-34 family includes 3 miRNAs encoded from 2 distinct genes: miR-34a has a transcript of its own, whereas miR-34b and miR-34c have the same transcript [106]. However, the sequences of these three members showed a high degree of identity. *miR-34a* and *miR-34c* have the same seed sequences, indicating that they have similar mRNA targets [107]. With the exception of the lung, the expression level of miR-34a is higher than that of miR-34b/c in the majority of human tissues [108]. Notably, deletions in the 1p36 region, where the gene encoding miR-34a resides, is often detected in most human cancers (such as rectal cancer, lung cancer, neuroblastoma, breast cancer, melanoma, etc.) [109]. Similarly, deletions in the 11q23.1 region, where the genes encoding miR-34b and miR-34c are located, are often observed in breast, prostate, cervical, and lung cancers [110]. In addition, frequent rearrangements such as translocations, insertions, and inversions in the 11q23 region have been detected in hematological malignancies [111]. The levels of *miR-34* family members are dysregulated in many types of cancer. Many studies have shown that the down-regulation of *miR-34* genes in tumor tissues when compared to healthy tissues is due to CpG methylation in various types of cancer (Figure 1.7) [112-114]. In addition, mature miR-34 was observed to be inactivated due to the lack of 5'-phosphate in several cancer cells [115]. Members of the *miR-34* family may also be useful in diagnosis, treatment, and prognosis of various tumors. In

summary, the dysregulation or inactivation of *miR-34a* and *miR-34b/c* genes are frequent events in tumorigenesis.

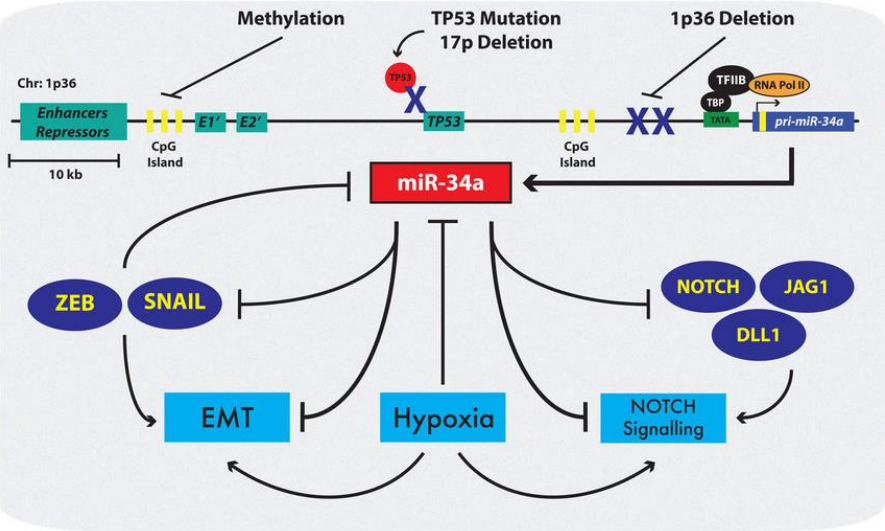


Figure 1. 7 Multiple mechanisms are utilized during tumorigenesis to reduce the levels and activity of miR-34a [116].

2.4.2 Role of miR-34 in CRC

Numerous studies have shown that levels of the *miR-34* family are lower in CRC tissue than in adjacent non-tumor tissues [117]. In human CRC cells, the level of miR-34a and miR-34c is down-regulated by their promoter hypermethylation [118]. Methylation of the *miR-34b/c* promoter was detected in up to 91.9% of CRC samples, and the methylation of the *miR-34a* promoter was detected in 45.1% of CRC samples. Low miR-34a expression is associated with elevated levels of SNAIL, c-Met, and beta-catenin expression, which is associated with metastasis to lymph nodes and the liver [119]. Epigenetic silencing of the *miR-34* family was observed in CRC cells, but not in normal colon epithelium. It is worth noting that the expression of *miR-34* family was restored after treatment with demethylating agents, which repressed invasion and metastasis in tumors [120]. Furthermore, the level of miR-34 was down-regulated in 36% of human CRC compared to normal tissues, which subsequently caused the E2F signal pathway activation and p53/p21 signal pathway suppression, enhancing proliferation of CRC cells and the development of CRC [94]. These miR-34 properties are mediated by various miR-34 target genes that regulate the cell cycle (*Cyclins D1/E2*, *CDK4/6*) [121], apoptosis (*Bcl-2*, *Survivin*, and *SIRT1*) [77,122], and EMT (*SNAIL* and *STAT3*) (Figure 1.8) [90,123].

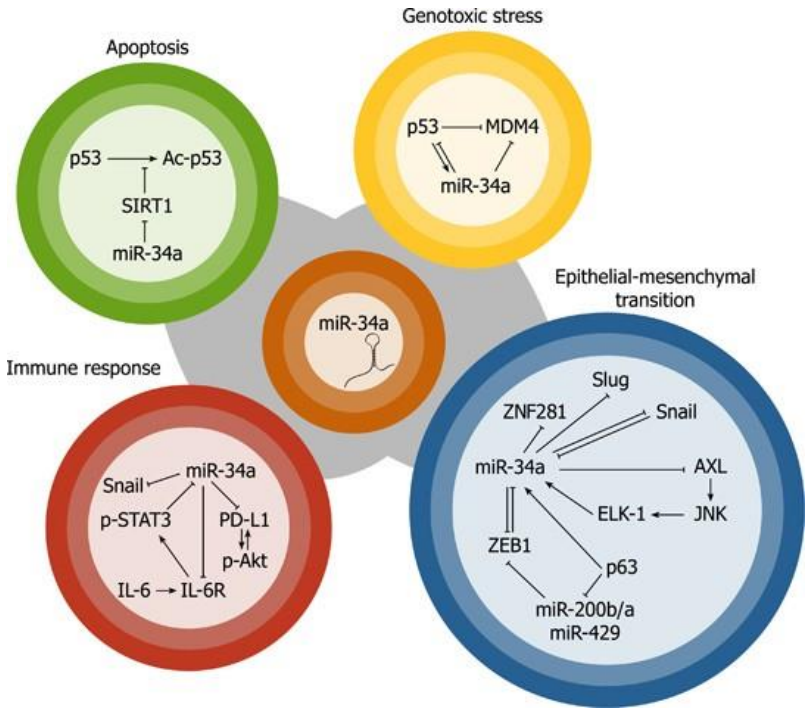


Figure 1.8 Molecular mechanism of miR-34 tumor suppression. Taken from [124].

2.4.2.1 MiR-34 and apoptosis

MiR-34 has important effect on apoptosis in many cancers by targeting anti-apoptotic or apoptotic genes. MiR-34a inhibits proliferation and induces apoptosis in SW480 and SW620 CRC cells through the targeting of SIRT1 [125]. SIRT1 regulates apoptosis in response to genotoxic and oxidative stress by acting as a NAD-dependent deacetylase. SIRT1 is downregulated by miR-34, which leads to increased expression of acetylated p53 as well as induction of p21 and PUMA, ultimately causing apoptosis in human CRC cells [122]. Ectopic expression of miR-34a can induce apoptosis and inhibit proliferation and invasion through suppressed expression of Bcl-2 and SIRT1. Interestingly, combined treatment with 5-FU amplifies this effect [126]. The miR-34a, miR-34b, and miR-34c levels are very low in NSCLC cells compared with normal cells, but PDGFRα and PDGFRα/β showed opposite trends. Garofalo et al. [127] reported that over-expression of miR-34a repressed the levels of PDGFRα and PDGFRα/β, thereby restoring TRAIL-induced apoptosis in NSCLC cell lines.

Sreetam et al. [128] used arsenic to induce ROS and DNA damage in hepatocytes, thereby causing the nuclear translocation of NRF2 and NF-kB and the activation of P53. They concluded that hepatitis is induced through a ROS/Nrf2/p53-miR-34a axis. It has been reported that *miR-34a* inactivation or mutation severely impairs p53-dependent apoptosis [82], whereas restoration of functional miR-34a has been shown to induce apoptosis and has chemo-sensitizing effects in various cancers [129]. Furthermore, studies showed that miR-34a inhibits the translation of HMGB1 (High-Mobility Group Box 1) to promote apoptosis and repress autoph-

agy in many cancers, including CRC, osteosarcoma, pancreatic cancer, etc.) [130,131]. In addition, miR-34a/b/c suppresses autophagy by regulating the expression of FOXM1 and ATG9A in CRC cells [132]. FOXM1 was also up-regulated by the combined inactivation of *microR-34a/b/c*, which promoted autophagy, suppressed apoptosis, and increased resistance to 5-FU. Consistently, silencing *ATG9A* or treatment with autophagy inhibitors restored 5-FU resistance in miR-34a/b/c-deficient cells.

2.4.2.2 miR-34 and EMT

During EMT epithelial characteristics are lost and mesenchymal characteristics and increased mobility are acquired. EMT is characterized by the downregulation of epithelial cell junctions (e.g. E-cadherin, occludin, and claudin) and up-regulation of interstitial adhesion proteins (e.g. vimentin, fibronectin, and N-cadherin). EMT is related to an aggressive or metastatic phenotype in CRC. The *miR-34* family is induced by p53 and regulates EMT by inhibiting EMT-TF, Notch signaling, Wnt signaling, and TGF-β signaling (Figure 1.9).

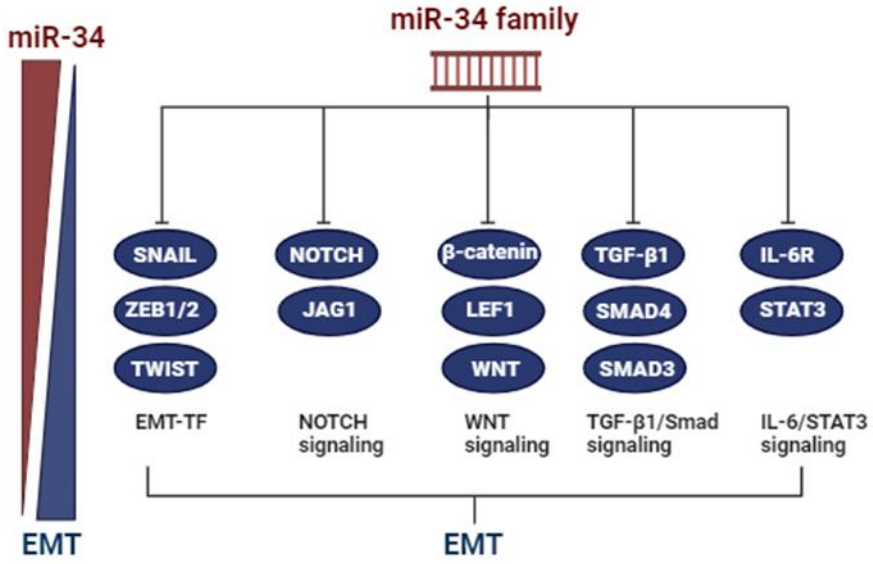


Figure 1.9 Mechanisms of EMT regulation by miR-34.

miR-34 binds to the 3'-UTR of EMT-TFs, regulating EMT. Overexpression of miR-34a mediates MET via down-regulation of SNAIL. Conversely, inhibition of miR-34a/b/c resulted in up-regulation of SNAIL, induced EMT markers and associated features, and promoted migration and invasion [123]. Conserved sequences matching with miR-34a are also found in the UTRs of *TWIST1*, *ZEB1*, and *ZEB2*, other EMT TFs. MiR-34 can directly bind stemness factors (such as c-MYC, CD44, CD133, and BMI1) and downregulate them [81,108,133]. Taken together, these studies demonstrate that miR-34a attenuates EMT by inhibiting EMT-TFs.

In addition, the *miR-34* family regulates EMT via the Wnt/β-catenin, TGF-β1/Smad3/4, and Notch-1 signaling pathways. LEF1 is a critical transcription factor in the Wnt/β-catenin signal pathway, that is central for regulating the proliferation and invasion of cancer cells. For example, miR-34a regulates the EMT process in prostate cancer cells by specifically inhibiting LEF1 [112]. Furthermore, miR-34a regulates *HOTAIR* to suppress the Wnt/β-catenin signaling pathway activation in gastric cancer cells [134]. Notch induces EMT during embryogenesis and promotes tumor metastasis. Luika et al. [135] demonstrated that Notch activates EMT by regulating the transcription of the Snail repressor in tumors. miR-34a inhibits the expression of fibronectin and vimentin and induces E-cadherin in CRCs by binding to the 3'-UTR of *Notch1* [117]. Notably, miR-34a can abrogate TGF-β-induced EMT, migration, and invasion via repressing Smad4 [136]. MiR-34b has repressed some critical components of the TGF-β signal pathway, such as TGF-βR1, and p-SMAD3 [137].

2.4.2.3 Clinical application of miR-34a therapy

Low expression of miR-34a/b/c is inversely related to the survival of patients with several cancer types in retrospective clinical studies [138]. Since miR-34 targets mRNAs encoding oncogenic factors, administration of miRNA mimics to restore the expression of tumor suppressor miRNAs that are inactivated or down-regulated in tumors may be an effective treatment for cancer patients [139-141]. Currently, miR-34a can be successfully delivered to mouse models by various methods (such as atelocollagen, liposome complexes, a class of 7C1 nanoparticles, and hyaluronic acid-chitosan nanoparticles). An orthotopic HCC (hepatocellular carcinoma) mouse model showed significant inhibiting of tumor growth and tumor regression in more than a third of the cases after treatment with MRX34 (liposomal formulation of miR-34a) [142]. Furthermore, miR-34a mimics have also repressed tumor growth in mouse models of lung cancer [143], lymphoma [144], and prostate cancer [145]. In a human phase I clinical trial, treatment with MRX34 showed evidence of anti-cancer activity in patients with solid tumors that were previously refractory to other types of previous treatments, and partial responses were observed in some patients with refractory solid tumors [146,147]. However, some patients experienced adverse events, even leading to the death of four patients. Presumably, the microRNAs were delivered to tumor cells but may cause lethal side effects in other organs or cell types. Although pharmacodynamic results have demonstrated the efficacy of miRNAs for the treatment of cancer, future miRNA-based therapies require the development of highly specific, safe, and efficient delivery vehicles. This is a problem also faced by other therapies that aim to restore genetic material lost or mutated in cancer cells.

2.5 Curcumin in CRC

2.5.1 The function of curcumin

Curcumin is a phytochemical extracted from *Curcuma longa*, a ginger-like plant. Curcumin was first isolated in 1815 by two German Scientists, Vogel and Pelletier [148]. It was obtained in crystalline form in 1870 and identified as [**1**,(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, or diferuloylmethane [149]. Since Aggarwal and colleagues reported the antitumor effects of curcumin in the 1990s [150], the anti-tumor mechanism of curcumin has received widespread attention from researchers. Curcumin is a natural phenol, yellow in color, and easily soluble in organic solvents (ethanol, propylene glycol, chloroform, etc.), but not easily soluble in water. Because of its hydrophobic properties, curcumin easily diffuses through the cell membrane into mitochondria, endoplasmic reticulum, and nucleus, where it can exert its effects. Due to hydrophobic properties, one of the common problems with administering curcumin is its poor bioavailability. To improve the solubility and bioavailability of curcumin, a variety of novel formulations including liposomes, nanoparticles, phospholipid complexes, and micelles have been developed [151-154].

The incidence of CRC varies widely between countries. The high incidence of colorectal cancer in Western countries may be related to life-style and diet. However, the much lower incidence of CRC in the Indian subcontinent has been suggested to be caused by the dietary consumption of turmeric [155]. Of note, although curcumin is poorly water-soluble, curcumin concentrations in colon tissue are relatively high compared with systemic blood levels and may benefit from oral treatment of colorectal cancer. Therefore, many studies have explored the effects of curcumin in CRC cells *in vitro*, animal models, and human trials (Figure 1.10).

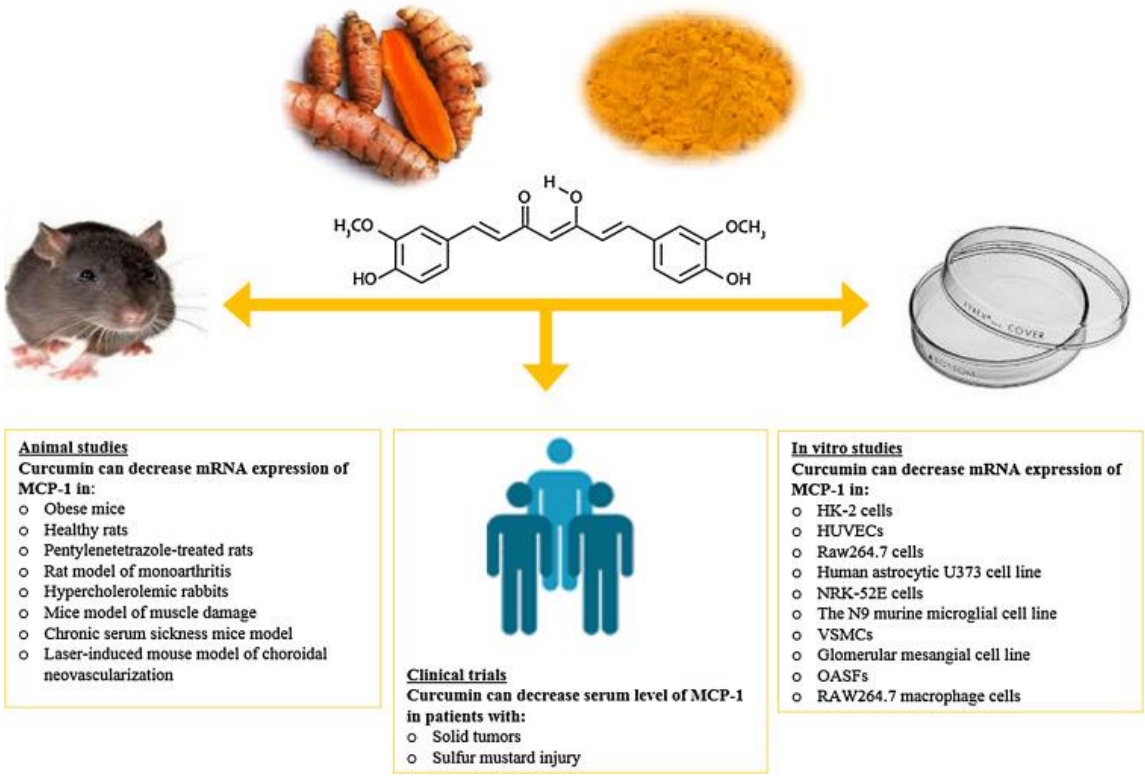


Figure 1.10 Effects of curcumin *in vitro, in vivo*, and in clinical studies. Taken from [156] .

2.5.1.1 Ex vivo studies: effects of curcumin on CRC cells

Based on many studies on cancer cells, curcumin inhibits cell proliferation by targeting multiple molecules and signaling cascades. In addition, curcumin is known to contribute to chemo-sensitization towards oxaliplatin, 5-FU, and irinotecan (Figure 1.11).

Curcumin is known to suppress the growth of CRC cells by disrupting the cell cycle and accelerating cell death. It has been reported to cause G1-phase arrest of the cell cycle and suppress cell proliferation in CRC via down-regulating the levels of CDK2 and cyclin D1 [157-159]. Besides, curcumin induced a G2/M-phase arrest of the cell cycle to promote apoptosis via up-regulating Wee1 and suppressing CDK1, CDC25c, and cyclin B1 [160]. Curcumin transactivates BAX and PUMA (Bcl-2 binding component 3) via the phosphorylation of p53 to induce cell death in CRC cells [161]. Notably, curcumin has a p53-independent pathway to induce cell cycle arrest and apoptosis of CRC cells [162-164]. Curcumin induces caspase-3-mediated apoptosis by reducing the level of mutant p53 in a dose- and time-dependent manner [165]. Besides, curcumin induces apoptosis in HCT-116 cells by inhibiting NF-kB and up-regulating DR5 [166]. Studies have found that curcumin directly activates Caspase 8 by cleaving and promoting Caspase 3 and Caspase 7, thereby triggering the Caspase cascade to execute apoptosis [167].

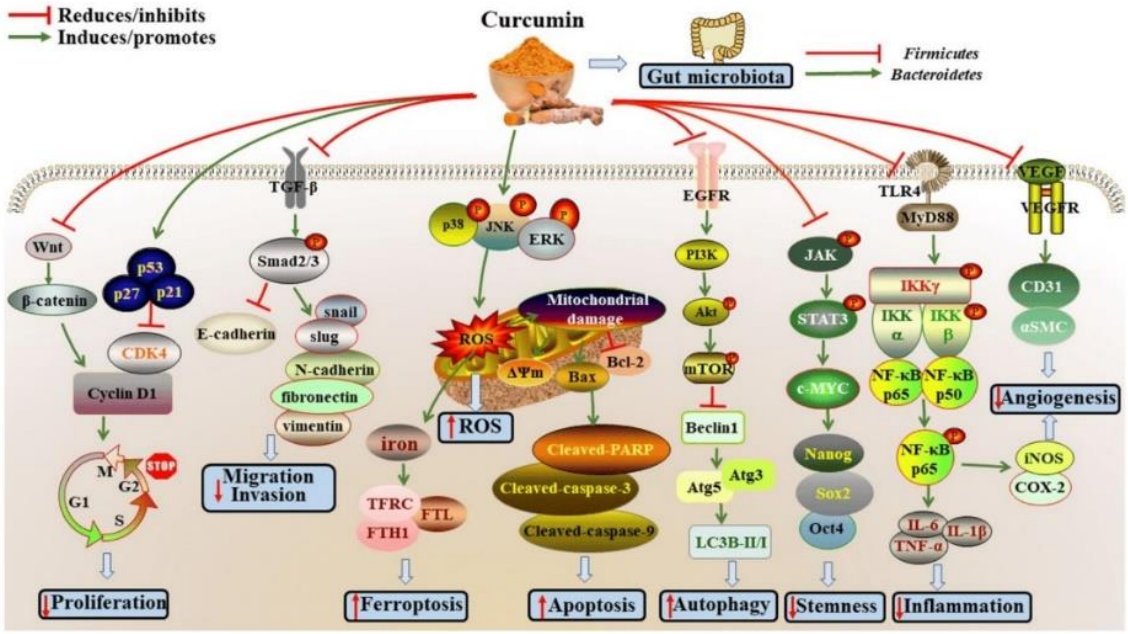


Figure 1.11 Schematic effect and mechanisms of curcumin on cancers [168].

Curcumin is known to exhibit both anti-oxidant and pro-oxidant characteristics, depending on its dosage. At low concentrations (⩽1 μM), curcumin has an antioxidant effect [169]. However, at higher concentrations (5 - 10 μM), curcumin mainly acts as an autophagy inducer by inhibiting the acetylation of cytoplasmic protein and regulating cell cycle arrest [170,171]. However, at higher concentrations of curcumin, autophagy is unable to rescue cells and leads to cell death [169].

Since cancer cells possess comparatively high ROS levels, they are more sensitive to oxidative stress induced by increased prooxidants and decreased antioxidants [172,173]. Curcumin regulates the cellular redox balance by disrupting mitochondrial homeostasis and enhancing cellular oxidative stress. Curcumin leads to increasing mitochondrial permeability, mitochondrial swelling, loss of membrane potential, and disruption of ATP synthesis by oxidizing thiols on the mitochondrial membrane, leading to malignant cell death. In addition, curcumin causes the death of cancer cells due to a significant increase of ROS by inhibiting ROS metabolism enzymes.

2.5.1.2 In vivo studies: effects of curcumin on CRC in animal models

In 2002, Perkins et al. [174] reported that ingestion of 0.2% curcumin (equivalent to 300 mg/kg) in animal models could significantly reduce the number of intestinal by 39% and prevent or delay the development of adenomas. The relevance of the result was further confirmed by Park et al [18]. Curcumin reduced mortality by 50% and completely prevented body weight loss in a mouse research model of AOM-induced colitis-associated colorectal cancer development. However, the chemo-preventive effect of curcumin appears to be indirectly related to

the normalizing effect of the colonic microbiota rather than its anti-inflammatory effect. Curcumin reduces tumor burden, improves survival, and normalizes the level of beta-catenin in CRC cells [175]. Furthermore, curcumin prevents CRC in DSS- and AOM-induced CRC mouse models via reversing DNA methylation of the gene encoding TNF (tumor necrosis factor) [176]. Ankur et al. [177] used 200 nm of CSSA NPs (redox-responsive chitosan/stearic acid nanoparticles) to deliver curcumin and DOX and exhibited therapeutic potential for colorectal cancer.

2.5.1.3 Human clinical studies

Although numerous preclinical studies showed that curcumin has anti-cancer and prevention effects on various cancers, it was not until 2001 that Cheng and colleagues reported a phase I study of curcumin in humans [178]. Curcumin has low bioavailability due to poor water solubility, in a group of 26 subjects, curcuminoids were detected in 29 out of 35 biopsy samples, after taking Cur-C3 (Curcumin C3 Complex) at 2.35 g per day for 14 days[179]. Therefore, curcumin can be bound and absorbed by colon cells. After taking 2 or 4 grams of curcumin for 30 days, patients had a reduction in the number of abnormal crypt lesions [180]. Cruz-Correa et al [181] showed that an oral mixture of 480 mg of curcumin with 20 mg of quercetin every day results in a 60% decrease in the number of colon polyps and a 50% decrease in polyp size, as detected by endoscopy six months later. In addition, in patients with advanced CRC refractory to standard chemotherapy, daily oral curcumin resulted in stable disease in 5 out of 15 patients after 4 months [182]. When patients with advanced colon cancer were supplemented with 2 g of curcumin per day, the overall survival rate in the curcumin combined with FOLFOX group was improved compared with the FOLFOX group [183]. Curcumin improves the quality of life of patients with stage III CRC by regulating the levels of ESR and CRP in serum [184]. Therefore, curcumin has been used in traditional chemotherapy as adjunctive treatment for advanced colorectal carcinoma with encouraging results.

2.5.2 Regulation of miR-34 by curcumin

Numerous studies have found that curcumin affects epigenetic alterations in CRC cells, such as histone modifications and DNA methylation. Furthermore, it regulates the expression of non-coding RNAs. A study by Roy et al. [118] reported that CDF (difluorinated curcumin), restores the level of miR-34a and miR-34c, which were downregulated in CRC cells, by demethylating the *miR-34a* and *miR-34c* promoters. Notably, the increase in the expression of miR-34 caused by CDF was independent of p53. In addition, CDF induces apoptosis and MET and represses stemness by up-regulating miR-34 and suppressing its target gene *Notch-1* [185] [186]. However, Shusuke et al. [187] reported that curcumin was only found to up-regulate the expression of miR-34 in *p53* WT CRC cells, but did not increase the level of miR-34 in *p53* mutant or *p53* null cell lines. However, they noted that the level of miR-34a target mRNAs was down-regulated in *p53* mutant/deficient CRC lines. Furthermore, curcumin showed a stronger inhibitory effect on the proliferation in HCT116 *p53* -/- cells than in

HCT116 *p53* +/+ cells. Therefore, studies on the regulation of miR-34 by curcumin have not yet achieved consistent results, indicating that further research is needed to fully understand its regulatory mechanism.

2.5.3 Curcumin-induced NRF2 activation

NRF2 is the product of the *NFE2L2* gene and possesses six highly conserved domains called NRF2-ECH homology (Neh) domains [188]. NRF2 is ubiquitously and constitutively expressed in cells to ensure rapid, protective cellular responses to inflammatory, oxidative, and metabolic stress. Under physiological conditions, NRF2 is ubiquitinated by the E3 ligase complex formed by CUL3/RBX1 (Cullin3 and RBX1 proteins), followed by NRF2 degradation via the proteasome 26S [189,190]. Curcumin, as an electrophilic molecule, causes the conformational change of KEAP1 by covalently modifying KEAP1-Cys-151, resulting in the release of NRF2 from the KEAP1-CUL3-RBX1 complex [191]. Curcumin markedly increased the level of HO-1(heme oxygenase-1), NRF2, p62/SQSTM1 protein, and its targets, and significantly reduced the levels of Keap1 to improve the response of therapies for cancer patients [192]. Furthermore, curcumin inhibits tumor growth by activating NRF2 expression, inducing endothelin B transcription, reducing ET-1 expression [193], and inhibiting Fen1 [194] expression. Curcumin increased the level of tumor suppressor p53 and up-regulated the levels of inflammatory mediator iNOS and down-regulated COX2 via activating NRF2 in the liver of lymphoma-bearing mice [195]. Curcumin acts as an NRF2 activator and exerts anti-cancer activity through multiple molecular mechanisms. However, hyperactivation of NRF2 has several advantages for cancer cells, including triggering cancer cell growth, proliferation, angiogenesis, and chemotherapy/radio-resistance. Likewise, NRF2 regulates the expression of various genes involved in cell proliferation and protein synthesis, including *Notch1*, *NPNT*, *VEGFC*, *IGF1*, *PHGDH*, *PSPH*, *PSAT1*, and *SHMT*, which is beneficial to cell proliferation [196,197]. Furthermore, the overactivation of NRF2 induced the expression of CD44 (cluster of differentiation 44), a transmembrane molecule that senses changes in the tumor microenvironment by linking with ECM components such as hyaluronic acid (HA) and promotes extracellular signaling and controls tumor development [198,199]. NRF2 serves as a key transcription factor in cells, and its pathway is considered to have dual functions in tumors (Figure 1.12). Therefore, precise identification of the molecular mechanisms involved in NRF2 expression and regulation will help cancer treatment.

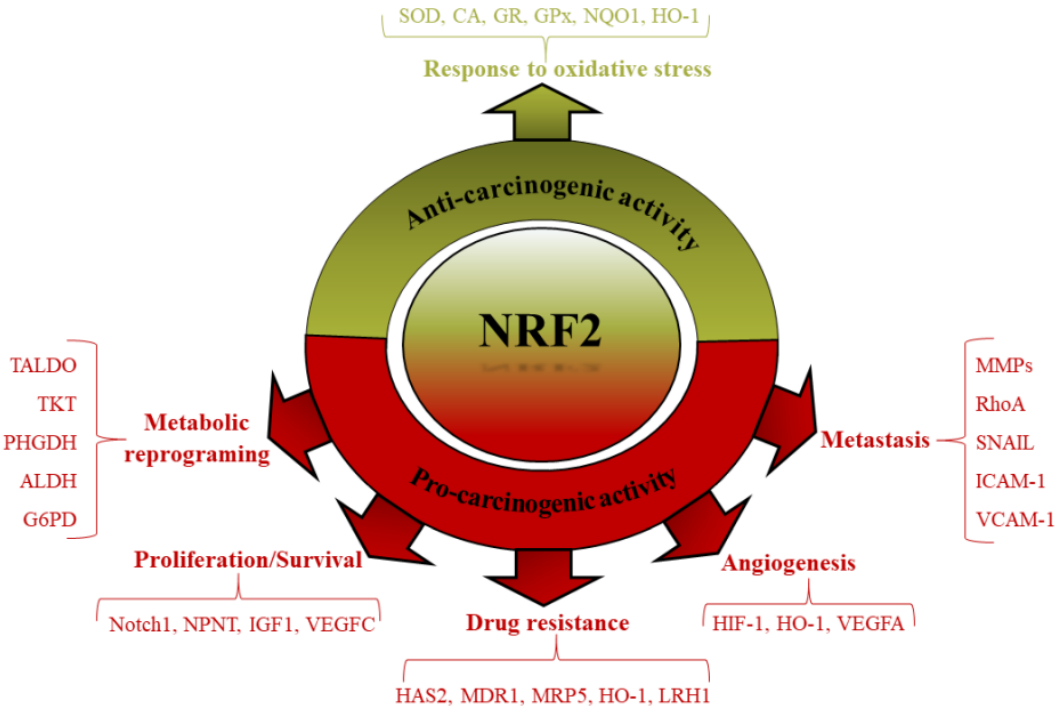


Figure 1.12 Anti/pro-carcinogenic activity of NRF2 [200].

2.6 Aspirin/Salicylate in CRC

2.6.1 The function of Aspirin/Salicylate

Aspirin is the most commonly used drug in the world and one of the most important pharmacological achievements of the 20th century. Aspirin has a fascinating history dating back more than 3,500 years to the use of willow extract to treat common fevers, pains, and inflammations. Salicin was first isolated from willow bark by Johann Andreas Buchner, a professor of pharmacology at the University of Munich, in 1828. Unfortunately, its irritation of the stomach and unpleasant taste greatly limit its use [201]. In 1897, Dr. Felix Hoffman, a German chemist acetylated the phenol group and produced pure stable ASA (acetylsalicylic acid) for the first time. Since then, ASA, also known as aspirin, has become the most widely used drug due to its positive anti-inflammatory effects and its role in reducing cancer risk, especially colon cancer.

Aspirin has a short half-period in the human bloodstream (15 - 20 min) and is quickly deacetylated and converted to salicylate *in vivo*. In plasma, the therapeutic concentration of salicylate used for therapeutic purposes is 15 -30 mg/dl (1.1 – 2.2 mmol/l) [202]. Aspirin is an unselective COX (Cyclooxygenase) inhibitor, which irreversibly inhibits COX-1 and COX-2. Xu et al.[203] found that aspirin and salicylate suppress COX-2 both at the mRNA and protein

levels. Upregulation of COX-2 and increased prostaglandin (PGE2) have been observed in the vast majority of colorectal cancers [204], and deletion of the COX-2 gene in a mouse model of FAP results in a reduction in the number and size of colorectal cancers [205]. Therefore, COX inhibitors have been used to reduce the risk of adenomas, colorectal cancer, and other cancers, and to prevent colorectal cancer recurrence (Figure 1.13).

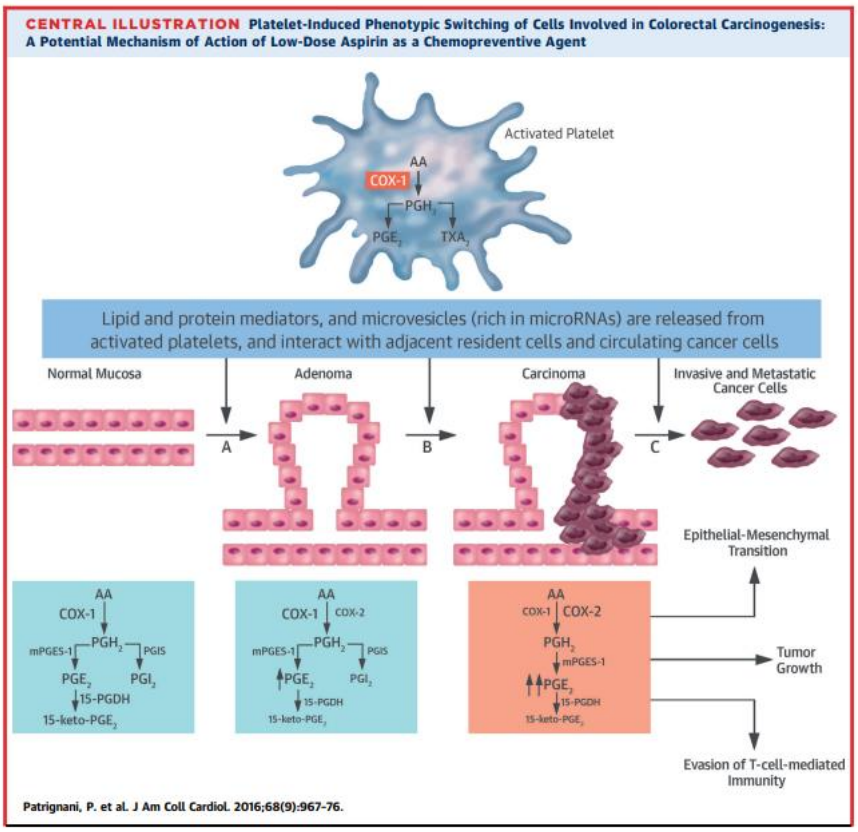


Figure 1.13 Potential mechanism of action of aspirin as a chemo-preventive agent. [206]

2.6.2 Aspirin/Salicylate in CRC

2.6.2.1 Ex vivo studies: effects of Aspirin/Salicylate on CRC cells

Experimental studies further elucidated the mechanisms by which the protective effects of NSAIDs are mediated (Figure 1.14). Aspirin exerts a direct effect on CRC cells and an indirect effect on the tumor microenvironment. Aspirin inhibits COX enzymes in epithelial and stromal cells to reduce prostaglandin synthesis, thereby inhibiting inflammation and cancer cell growth [207]. NF-κB is a transcription factor that stimulates the expression of anti-apoptotic genes and usually exists as a heterodimeric complex bound in the cytoplasm through the inhibitor protein IKB [208]. Aspirin and salicylic acid have been found to suppress IKK to prevent the translocation of NF-κB to the nucleus [209]. Therefore, aspirin may cause retention of NF-

κB protein in the cytoplasm and inhibit the transcription of anti-apoptotic genes. In addition, aspirin can prevent EMT by inhibiting the expression of TLR-4 and inhibiting the activation of the NF-κB signaling pathway [210]. Aspirin can activate AMPK by modifying the AMP: ATP ratio in cells and then inhibits the activity of the mTOR signalling pathway [211]. Activation of the Wnt/β-catenin pathway is the first step in nearly all colorectal cancers (CRC) [212]. Stable β-catenin translocates to the nucleus and binds to members of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family of transcription factors, and then activates transcription of Wnt target genes such as *cyclin D* and *Myc* [213]. Aspirin acts by inhibiting PP2A, leading to increased levels of phosphorylated β-catenin and its degradation, which reduces the activity of the Wnt/β-catenin pathway and the mRNA and protein levels of c-MYC [214,215]. In addition, aspirin also has been shown to directly acetylate wild-type and mutant p53, suggesting that aspirin exerts its tumor suppressive effects by restoring p53 function [216] .

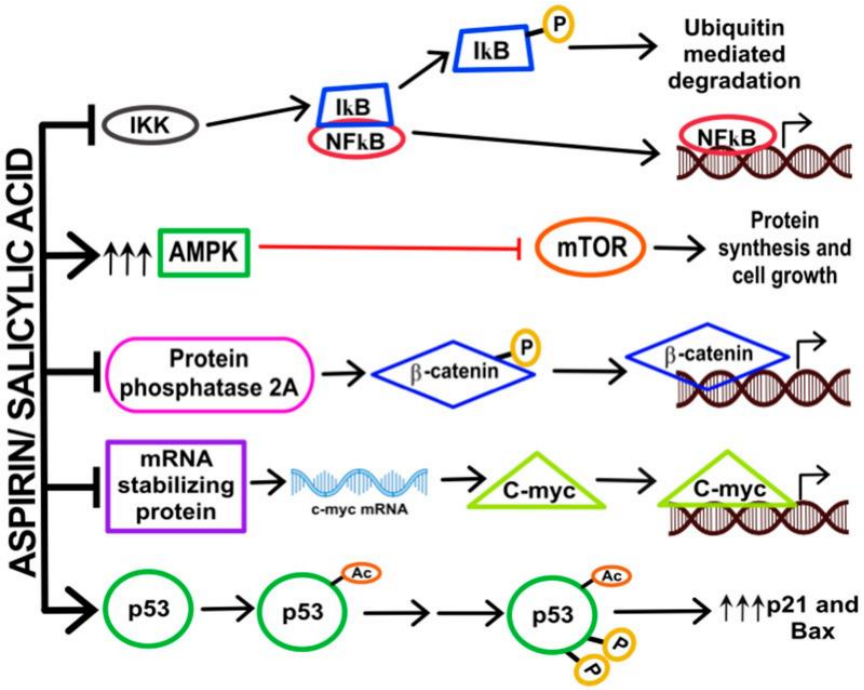


Figure 1.14 The anti-cancer mechanism of aspirin/salicylate [207].

The indirect mechanism by which aspirin affects the tumor microenvironment is through the inhibition of platelets. Platelets are activated in the tumor microenvironment after interacting with epithelial cancer cells. Activated platelets produce soluble growth factors and angiogenic factors and enhance COX-2 expression in stromal cells and endothelial cells in the tumor microenvironment. Although aspirin has a short half-life, it exerts an irreversible effect on COX-1, which cannot be regenerated in platelets due to the lack of nuclei [217]. Therefore, the inhibition of COX-1 in platelets and COX-2 in epithelial cells is one of the mechanisms by which aspirin inhibits platelet-mediated tumorigenesis. Several studies have shown

that aspirin downregulates c-MYC levels and inhibits cell proliferation in various cancer entities [218]. Aspirin also blocks TGF-β secretion from activated platelets. TGF-β inhibits the activation and function of NK cells and promotes the survival of tumor cells in the blood circulation [219].

2.6.2.2 In vivo study: effects of Aspirin/Salicylate on CRC animal models

Many studies have proven that the intestinal microbiota is a key factor in CRC initiation and progression. After being fed with aspirin or other NSAIDs fecal samples of mice showed enrichment of beneficial bacteria (e.g. Lactobacillus genera and Bifidobacterium) and a reduction of pathogenic microbes (e.g. Fusobacterium nucleatum, Alistipes Finegoldii, and Bacteroides fragile) which may contribute to reduced tumor formation in mice [220 221].

Many cases of CRC have associated with environmental factors rather than heritable, genetic alterations. Risks involve food-borne and environmental mutagenicity, specific commensal bacteria and pathogens in the intestine, and chronic inflammation of the intestine preceding tumorigenesis. In addition to intestinal bacteria, cytokines, immune cells, and other immune mediators are involved in nearly all steps of the development of colon tumors, including initiation, facilitation, metastasis, and progression. Aspirin is an anti-inflammatory drug, which was reported to prevent CRC via inhibiting H3K27ac enrichment at the promoters of *TNF-α*, *iNOS*, and *IL-6* in the AOM/DSS-induced CRC mouse model [222]. Bousserouel et al. [223] showed that aspirin suppresses the level of prostaglandin E2 (PGE2), soluble mediators of inflammation (TNF-α and lL-1β), and metalloproteinase (MMP7 and MMP3).

In addition, intermittent therapy with naproxen and aspirin inhibits azoxy-methane-induced progression of colon adenomas to adenocarcinoma and invasive carcinoma [224]. In other studies, aspirin or other NSAIDs have been reported to prevent CRC in mouse models and exhibit anti-proliferative and pro-apoptotic effects *in vitro*. In addition, NOSH-ASA (NOSH-Aspirin, a novel nitric oxide- and hydrogen sulfide-releasing hybrid) decreased the volume of tumors by 85% in a mouse model of human CRC cells xenografts [225].

2.6.2.3 Human clinical studies of Aspirin/Salicylate

The use of NSAIDs for the treatment of CRC has been extensively studied, with evidence supporting a reduction in CRC incidence following aspirin therapy. Regularly intake of aspirin and other NSAIDs has demonstrated effective anti-cancer effects when taken for 5 years or longer [226-229]. A meta-analysis of 17 case-control studies involving over 12,000 CRC cases revealed a decreased risk of CRC with regular aspirin intake (OR = 0.52, 95% CI 0.58-0.67). Furthermore, another meta-analysis of four random controlled trials encompassing more than 14,000 patients with CRC found that daily aspirin treatment at doses of 75 to 300 mg for 5 years or more reduced the long-term risk of CRC by 24% [227]. Interestingly, the results from epidemiological and clinical studies suggest that low-dose (75 to 81 mg) is as effective as high-dose aspirin (⩾325 mg) in preventing CRC [227].

CRC typically originated as non-malignant adenomatous polyps that progress to adenomas and ultimately develop into invasive cancer. Following aspirin intervention in FAP patients, both the size and number of intestinal polyps were significantly reduced, suggesting aspirin's potential to mitigate colorectal adenoma development in FAP patients [230]. This led the USPSTF to endorse aspirin as a primary preventive measure against CRC in 2015 [27]. Similarly, aspirin intake has been associated with reduced risks of breast cancer, oesophageal, biliary, and gastric cancer. Moreover, multiple studies have demonstrated reduced mortality associated with CRC when aspirin was taken after diagnosis with CRC [231]. A meta-analysis of 27 trials involving over 230,000 CRC patients revealed that taking aspirin post-diagnosis was correlated with improved overall survival [232]. Patients with colorectal cancer were also less likely to develop advanced stages of the diseases, suggesting aspirin may inhibit CRC progression [233]. Consequently, aspirin is currently regarded as one of the most promising substances for CRC prevention and suppression of CRC progression [27].

Although the standard treatment for patients with CRC is complete surgical resection, approximately 50% of patients with regional node-positive disease (stage 3) relapse after surgical treatment within the first 5 years. Long-term aspirin therapy after CRC resection reduces the risk of recurrence and improves overall survival, particularly in patients with *PIK3CA*-mutated tumors [231 234].

2.6.3 AMPK activation by Aspirin/Salicylate

AMPK is a cellular energy sensor conserved in all eukaryotes [230]. When cells are stressed, AMPK phosphorylates targets, inhibiting the ATP (adenosine triphosphate) consumption process and promoting catabolic pathways for ATP production to restore energy homeostasis[235]. Phosphorylation of Thr172 in the AMPK alpha subunit by LKB1 or CaMKKβ (Ca^{2+}-dependent kinase) significantly activates AMPK (>100-fold) [235,236]. Additionally, binding of AMP (but not ATP) further allosterically activates the phosphorylated kinase up to 10-fold [226]. Most medicines or xenobiotics activate AMPK by synthesis of ATP in the mitochondria and increasing the levels of AMP and ADP [237]. However, A-769662 (a synthetic activator) induces allosteric activation and inhibits dephosphorylation of Thr172 by directly binding to several sites of AMPK [238-240].

Salicylate binds to the same site on AMPK as the synthetic activator (A-769662). As a direct AMPK activator, salicylate binds to the opposite side of the β-CBM, in the gap between β-CBM and α-KDN N-lobes, inducing allosteric activation and preventing the dephosphorylation of Thr172 [241]. Salicylates at concentrations of 1 mM and above can have significant effects on AMPK activation [242]. The general therapeutic plasma concentration range of salicylates is 15 - 30 mg/dl (1.1 - 2.2 mmol/l) [202]. At these concentrations, AMPK can be activated by salicylates.

2.6.4 The AMPK/NRF2 connection

NRF2 is a transcription factor involved in stress responsiveness, aiding in the resistance against xenobiotic, oxidative, and proteotoxic insults. AMPK is a key regulator of cellular energy homeostasis. It not only regulates metabolism to provide adequate ATP for cells but also regulates inflammation and redox balance. Due to the overlap in the regulatory cellular responses after activation of AMPK and NRF2, as well as the common stressors that activate AMPK and NRF2 signaling pathways, it is reasonable to hypothesize that the AMPK and NRF2 signaling pathways may be interdependent and cooperate to jointly regulate cellular homeostatic states (Figure 1.15) [243,244].

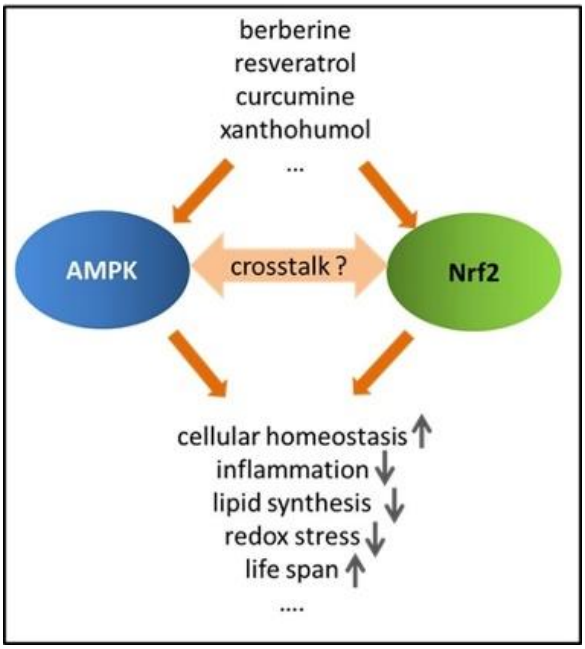


Figure 1.15 Potential crosstalk between the AMPK and NRF2 signaling pathways [245].

Metformin regulates aging and extends the life-span of C. elegans via activating SKN-1/Nrf2, AMPK, and LKB1 [246]. Joo and colleagues were the first to discover that AMPK directly phosphorylates NRF2 at serine 558 [247]. Additionally, AMPK suppresses GSK3β through phosphorylation of serine 9. This AMPK-mediated inhibition of GSK3β reduced βTrCP-triggered degradation and/or nuclear exclusion of NRF2 [243,244]. Furthermore, Liu et al.[248] demonstrated that the AMPK activator (AICAR) induces the expression *HO-1* (heme oxygenase-1), which encodes an antioxidative enzyme, in endothelial cells through activating NRF2. They also observed that the level of NRF2 rapidly increased after treatment with AICAR, as an AMPK-dependent process. Mo et al. [249] observed a similar activation of NRF2 by Berberine, in an AMPK-dependent way. In contrast, treatment with the AMPK inhibitor Dorsomorphin resulted in decreased the nuclear levels of NRF2 [250].

Keap1 servers as an inhibitor of NRF2, binding and redirecting NRF2 to proteasomal degradation, while also preventing its nuclear translocation. Notably, certain compounds such

as TBHQ, sulforaphane, and quercetin have been confirmed to induce NRF2 activation by modifying cysteine residues in Keap1, while also activating AMPK. Additional studies found that AMPK affects the affinity of p62 for KEAP1 through phosphorylation and promotes p62-assisted KEAP1 depletion through enhanced autophagy, thus stabilizing NRF2 [251-253]. Therefore, AMPK and NRF2 signaling pathways may be able to crosstalk via Keap1. Overall, activated AMPK enhances NRF2 signaling, while NRF2 in turn negatively feeds back with a delay by restoring metabolic and redox balance, thereby inhibiting AMPK signaling.

2.6.5 The c-MYC/NRF2 connection

C-MYC and Bach1 are the two main inhibitors of NRF2, thereby hindering NRF2-mediated adaptation. c-MYC as a transcription factor, activates the expression of genes encoding factors, which enhance cellular processes associated with proliferation. Davis et al. [254] found that binding of c-MYC to NRF2, not only abrogated NRF2-mediated transcription but also reduces the half-life of NRF2 protein by inducing the degradation of NRF2. Silencing of *c-MYC* activated NRF2 by reducing the substitution of NRF2 by c-MYC in the EpRE complex and/or c-MYC-mediated NRF2 degradation. C-MYC regulates the EpRE/NRF2 signaling pathway by interacting with the EpRE binding complex and increasing the degradation of NRF2 (Figure 1.16). Furthermore, c-MYC, NRF2 and c-Jun form a ternary complex and reduce the stability of NRF2. Conversely, the amount of c-MYC protein in the nucleus was decreased after treatment with NRF2 activators (such as HNE and tBHQ) [255 256]. In summary, there appears to be a regulatory link between c-MYC and NRF2 in cancer that deserves further exploration.

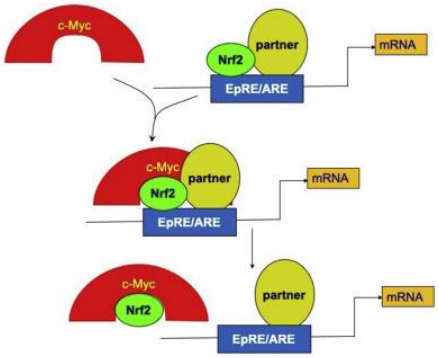


Figure 1.16 C-MYC-mediated inhibition of NRF2 function [254].

2.7 Aims of study

2.7.1 Analysis of the role of miR-34 in the suppression of CRC by curcumin

(1) Determine whether the tumor suppressing properties of curcumin in CRC cells are p53- or miR-34-dependent or independent.

(2) Analyze the mechanism of miR-34 induction by curcumin.

(3) Determine the relevance of miR-34 in curcumin-induced apoptosis, senescence, and prevention of metastasis in CRC cells.

(4) Determine whether p53 and/or miR-34 modulate the sensitivity towards curcumin and/or 5-FU.

2.7.2 Analysis of the role of miR-34 in CRC suppression by salicylate

(1) Determine whether the tumor suppressing properties of salicylate in CRC cells are p53- or miR-34a dependent or independent.

(2) Determine the relevance of miR-34 for salicylate-induced apoptosis, and inhibition of EMT and metastasis in CRC cells.

(3) Analyze the mechanism of miR-34 induction by salicylate.

(3) Determine and characterize the mechanism of NRF2 activation by salicylate.

(4) Analysis of the regulatory effects of c-MYC on NRF2 and miR-34 in CRC cells.

3. Summary (in Englisch)

3.1 Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis

Curcumin is a polyphenol extracted from the rhizome of the turmeric plant (Curcuma longa) and has been used for centuries in traditional Chinese and Ayurvedic medicine. Notably, curcumin has been shown to have the potential to support the prevention and treatment of CRC in preclinical and clinical studies. However, the exact mechanism of action and downstream mediators of curcumin's tumor suppressive effects have remained largely unknown. The purpose of our study was to determine the role of *miR-34a and miR-34b/c* in the tumor suppressive functions of curcumin. Therefore, we treated three isogenic CRC cell lines with CRISPR/Cas9-mediated inactivation of *p53, miR-34a, and/or miR-34b/c* genes with curcumin and analyzed various cell processes, including proliferation, apoptosis, and DNA damage. Furthermore, we analyzed the mechanism of *miR-34* induction by curcumin by using biochemical methods, such as Western blot, qCHIP, and qPCR analysis. Finally, we utilized a mouse model of metastasis to determine the effects of miR-34 and curcumin on the metastatic potential of CRC cells.

Here, we found that curcumin induced senescence and apoptosis, and inhibited migration, invasion, and metastasis in a *p53*-independent manner in CRC cells. After treatment with curcumin CRC cells showed an accumulation of ROS, while the antioxidant N-acetylcysteine (NAC) inhibited curcumin-induced apoptosis. Therefore, the accumulation of ROS may mediate curcumin-induced apoptosis.

Furthermore, curcumin activated the KEAP1/NRF2 pathway in CRC cells by the generation of ROS. In the absence of stress, NRF2 is rapidly degraded by the KEAP1-CUL3-RBX1 complex and sequestered in the cytoplasm to maintain low basal levels of NRF2. Under oxidative stress, electrophiles, and ROS change the conformation of KEAP1 and cause the release of NRF2 from the KEAP1-CUL3-RBX1 complex. NRF2 subsequently trans-locates to the nucleus, where it binds to ARE motifs near the promoters of its target genes, which encode mediators of antioxidative response. The antioxidant NAC inhibited curcumin-induced apoptosis and largely reversed ROS-induced nuclear translocation and activation of NRF2. Noteworthy, curcumin induced the expression of *miR-34a and miR-34b/c*, in a ROS/NRF2-dependent and *p53*-independent manner. We showed that NRF2 directly binds to the promoter region of *miR-34a and miR-34b/c*. NAC treatment and *NRF2*-specific siRNA abolished the induction of *pri-miR-34a* and *pri-miR34b/c* by curcumin. Thus, our results indicate that the *miR-34a and miR-34b/c* genes are an integral part of the NRF2-mediated oxidative stress response program. Deletion of the *miR-34a and miR-34b/c* genes significantly

decreased curcumin-induced senescence and apoptosis, and diminished the effects of curcumin or ectopic NRF2 on migration and invasion. Curcumin induced mesenchymal-to-epithelial transition (MET) of CRC cells and prevented lung metastasis formation of CRC cells in mice in a *miR-34a*-dependent manner. Finally, we showed that curcumin may increase the therapeutic effects of 5-FU in *miR-34a/b/c- and/or p53*-deficient CRC cells. Altogether, we demonstrated that the mechanism of tumor suppression by curcumin involves the activation of the KEAP1/NRF2/miR-34a/b/c pathway.

3.2 Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cascade resulting in suppression of colorectal cancer metastasis

Substantial evidence supports the potential role of aspirin and its active metabolite salicylates in cancer prevention, particularly in the chemoprevention of colorectal cancer. Moreover, aspirin may reduce the risk of progression from established CRC to advanced colorectal cancer, as well as metastasis and mortality. Although aspirin has a well-established role in cancer prevention, the underlying molecular mechanisms are not fully understood. Here we characterized the activation of an AMPK-NRF2-*miR-34a/b/c* axis by salicylate as a novel mechanism of salicylate-mediated suppression of CRC. Salicylate activated AMPK, thereby activating NRF2, which directly induced *miR-34a/b/c* expression. We used CRISPR/Cas9-mediated inactivation of *miR-34a and/or miR-34b/c* to determine the involvement of miR-34 in salicylate-induced tumor suppression. We further analyzed the role of AMPK and MYC in these regulations. Finally, we used a tail vein mouse model of metastasis to analyze to role of salicylate and miR-34a in metastasis.

In the study mentioned above, we found that the activation of NRF2 by curcumin is mediated through the induction of ROS. Here, we showed that salicylate also activates NRF2, but in a ROS and also *p53*-independent manner. Instead, we showed that salicylate activates NRF2 via AMPK. Since ROS may cause DNA damage, leading to cell death or the induction of mutations, not only in cancer cells but also in normal cells, using salicylates as chemopreventive drugs may have fewer side effects than curcumin. We showed that salicylate upregulates *miR-34a and miR-34b/c* expression in a *p53*-independent way. Our results demonstrate that AMPK α1 and AMPK β1 subunits are important for salicylate induction of *miR-34a/b/c*, as inhibition of either subunit prevents activation of NRF2 and induction of *miR-34a/b/c*. In addition, salicylate suppressed c-MYC both at the mRNA and protein levels independent of *p53*. Inhibition of *AMPK* by siRNA or chemical inhibitors eliminated the inhibition of c-MYC by salicylate, showing that salicylate inhibits c-MYC expression through AMPK. This suppression of c-MYC was necessary for NRF2-mediated induction of *miR-34a/b/c*, since ectopic expression of *c-MYC* prevented activation of NRF2 and the induction of *miR-*

34a/b/c by salicylate. Furthermore, salicylate inhibited proliferation, migration, invasion, EMT, and metastasis and induced apoptosis in CRC cells in a *miR-34a/b/c*-dependent manner. Furthermore, the experimental suppression of miR-34a largely abrogated the tumor suppressing effects of salicylate on metastases formation in mice. Altogether, our results demonstrate that the NRF2/*miR-34a* and *miR-34b/c* axis mediates the effects of salicylate on cell viability, apoptosis, migration, invasion, MET, and metastasis.

4. Zusammenfassung (deutsch)

4.1 Curcumin aktiviert eine ROS/KEAP1/NRF2/miR-34a/b/c-Kaskade, um die Metastasierung von Darmkrebs zu unterdrücken

Curcumin ist ein Polyphenol, das aus dem Rhizom der Kurkumapflanze (Curcuma longa) gewonnen wird und seit Jahrhunderten in der traditionellen chinesischen und ayurvedischen Medizin verwendet wird. Insbesondere wurde in präklinischen und klinischen Studien gezeigt, dass Curcumin das Potenzial hat, die Prävention und Behandlung von Darmkrebs zu unterstützen. Der genaue Wirkmechanismus und die nachgeschalteten Mediatoren der tumorsuppressiven Wirkung von Curcumin sind jedoch weitgehend unbekannt. Der Zweck unserer Studie bestand darin, die Rolle von *miR-34a* und *miR-34b/c* bei den tumorsuppressiven Funktionen von Curcumin zu bestimmen. Daher haben wir drei isogene CRC-Zelllinien mit CRISPR/Cas9-vermittelter Inaktivierung der *p53*-, *miR-34a*- und/oder *miR-34b/c*-Gene mit Curcumin behandelt und verschiedene Zellprozesse analysiert, darunter Proliferation, Apoptose und DNA-Schäden. Darüber hinaus analysierten wir den Mechanismus der *miR-34*-Induktion durch Curcumin mithilfe biochemischer Methoden wie Western Blot, qCHIP und qPCR-Analyse. Schließlich verwendeten wir ein Mausmodell der Metastasierung, um die Auswirkungen von miR-34 und Curcumin auf das Metastasierungspotenzial von CRC-Zellen zu bestimmen.

Hier fanden wir heraus, dass Curcumin Seneszenz und Apoptose induzierte und Migration, Invasion und Metastasierung in CRC-Zellen auf *p53*-unabhängige Weise hemmte. Nach der Behandlung mit Curcumin zeigten CRC-Zellen eine Akkumulation von ROS, während das Antioxidans N-Acetylcystein (NAC) die Curcumin-induzierte Apoptose hemmte. Daher kann die Akkumulation von ROS eine Curcumin-induzierte Apoptose vermitteln.

Darüber hinaus aktivierte Curcumin den KEAP1/NRF2-Signalweg in CRC-Zellen durch die Erzeugung von ROS. In Abwesenheit von Stress wird NRF2 durch den KEAP1-CUL3-RBX1-Komplex schnell abgebaut und im Zytoplasma sequestriert, um niedrige Basalspiegel von NRF2 aufrechtzuerhalten. Unter oxidativem Stress verändern Elektrophile und ROS die Konformation von KEAP1 und bewirken die Freisetzung von NRF2 aus dem KEAP1-CUL3-RBX1-Komplex. Anschließend wandert NRF2 in den Zellkern, wo es an ARE-Motive in der Nähe der Promotoren seiner Zielgene bindet, die Mediatoren der antioxidativen Reaktion kodieren. Das Antioxidans NAC hemmte die Curcumin-induzierte Apoptose und kehrte die ROS-induzierte nukleare Translokation und Aktivierung von NRF2 weitgehend um. Bemerkenswert ist, dass Curcumin die Expression von miR-34a und miR-34b/c auf ROS/NRF2-abhängige und *p53*-unabhängige Weise induzierte. Wir haben gezeigt, dass

NRF2 direkt an die Promotorregion von miR-34a und miR-34b/c bindet. NAC-Behandlung und *NRF2*-spezifische siRNA hoben die Induktion von *pri-miR-34a* und *pri-miR34b/c* durch Curcumin auf. Somit deuten unsere Ergebnisse darauf hin, dass die Gene miR-34a und miR-34b/c ein integraler Bestandteil des NRF2-vermittelten Reaktionsprogramms auf oxidativen Stress sind. Die Löschung der Gene *miR-34a* und *miR-34b/c* verringerte die Curcumin-induzierte Seneszenz und Apoptose signifikant und verringerte die Auswirkungen von Curcumin oder ektopischem NRF2 auf Migration und Invasion. Curcumin induzierte den mesenchymalen zum epithelialen Übergang (MET) von CRC-Zellen und verhinderte die Bildung von Lungenmetastasen von CRC-Zellen bei Mäusen auf miR-34a-abhängige Weise. Schließlich haben wir gezeigt, dass Curcumin die therapeutische Wirkung von 5-FU in *miR-34a/b/c*- und/oder *p53*-defizienten CRC-Zellen verstärken kann. Insgesamt haben wir gezeigt, dass der Mechanismus der Tumorsuppression durch Curcumin die Aktivierung des KEAP1/NRF2/miR-34a/b/c-Signalwegs beinhaltet.

4.2 Salicylat induziert AMPK und hemmt c-MYC, um eine NRF2/ARE/miR-34a/b/c-Kaskade zu aktivieren, was zur Unterdrückung der Metastasierung von Darmkrebs führt

Umfangreiche Belege belegen die potenzielle Rolle von Aspirin und seinen aktiven Metaboliten Salicylaten bei der Krebsprävention, insbesondere bei der Chemoprävention von Darmkrebs. Darüber hinaus kann Aspirin das Risiko des Fortschreitens von etabliertem Darmkrebs zu fortgeschrittenem Darmkrebs sowie von Metastasen und Mortalität verringern. Obwohl Aspirin eine etablierte Rolle in der Krebsprävention spielt, sind die zugrunde liegenden molekularen Mechanismen nicht vollständig verstanden. Hier haben wir die Aktivierung einer AMPK-NRF2-*miR-34a/b/c*-Achse durch Salicylat als einen neuen Mechanismus der Salicylat-vermittelten Unterdrückung von CRC charakterisiert. Salicylat aktivierte AMPK und aktivierte dadurch NRF2, was direkt die *miR-34a/b/c*-Expression induzierte. Wir nutzten die CRISPR/Cas9-vermittelte Inaktivierung von *miR-34a* und/oder *miR-34b/c*, um die Beteiligung von *miR-34* an der Salicylat-induzierten Tumorsuppression zu bestimmen. Wir haben die Rolle von AMPK und MYC in diesen Vorschriften weiter analysiert. Schließlich verwendeten wir ein Schwanzvenen-Mausmodell der Metastasierung, um die Rolle von Salicylat und miR-34a bei der Metastasierung zu analysieren.

In der oben erwähnten Studie haben wir herausgefunden, dass die Aktivierung von NRF2 durch Curcumin durch die Induktion von ROS vermittelt wird. Hier haben wir gezeigt, dass Salicylat auch NRF2 aktiviert, jedoch auf ROS- und auch *p53*-unabhängige Weise. Stattdessen haben wir gezeigt, dass Salicylat NRF2 über AMPK aktiviert. Da ROS nicht nur in Krebszellen, sondern auch in normalen Zellen DNA-Schäden verursachen und zum Zelltod oder zur Induktion von Mutationen führen können, hat die Verwendung von Salicylaten als

chemopräventive Medikamente möglicherweise weniger Nebenwirkungen als Curcumin. Wir haben gezeigt, dass Salicylat die Expression von miR-34a und miR-34b/c auf *p53*-unabhängige Weise hochreguliert. Unsere Ergebnisse zeigen, dass die Untereinheiten AMPK α1 und AMPK β1 für die Salicylat-Induktion von *miR-34a/b/c* wichtig sind, da die Hemmung einer der beiden Untereinheiten die Aktivierung von NRF2 und die Induktion von miR-34a/b/c verhindert. Darüber hinaus unterdrückte Salicylat c-MYC unabhängig von p53 sowohl auf mRNA- als auch auf Proteinebene. Die Hemmung von *AMPK* durch siRNA oder chemische Inhibitoren beseitigte die Hemmung von c-MYC durch Salicylat, was zeigt, dass Salicylat die c-MYC-Expression durch AMPK hemmt. Diese Unterdrückung von c-MYC war für die NRF2-vermittelte Induktion von miR-34a/b/c notwendig, da die ektopische Expression von c-MYC die Aktivierung von NRF2 und die Induktion von miR-34a/b/c durch Salicylat verhinderte. Darüber hinaus hemmte Salicylat Proliferation, Migration, Invasion, EMT und Metastasierung und induzierte Apoptose in CRC-Zellen auf miR-34a/b/c-abhängige Weise. Darüber hinaus wurde durch die experimentelle Unterdrückung von miR-34a die tumorunterdrückende Wirkung von Salicylat auf die Metastasenbildung bei Mäusen weitgehend aufgehoben. Insgesamt zeigen unsere Ergebnisse, dass die NRF2/miR-34a- und miR-34b/c-Achse die Auswirkungen von Salicylat auf die Lebensfähigkeit der Zellen, Apoptose, Migration, Invasion, MET und Metastasierung vermittelt.

5. Paper I

Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis

Chunfeng Liu, Matjaz Rokavec, Zekai Huang and Heiko Hermeking. Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis. Cell Death and Differentiation, 2023.30(7): p.1771-1785. DOI: 10.1038/s41418-023-01178-1

6. Paper II

Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cas-cade resulting in suppression of colorectal cancer metastasis

Chunfeng Liu, Matjaz Rokavec, Zekai Huang and Heiko Hermeking. Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cascade resulting in suppression of colorectal cancer metastasis. Cell Death and Disease. 2023 Oct 28;14(10): 707. DOI: 10.1038/s41419-023-06226-9

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