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AG Molekulare Onkologie und Immuntherapie

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# Identification of the microsatellite stability status and inhibitor of NF-kappa-B expression levels as biomarkers to predict sensitivity to pharmaceutical curcumin in colorectal cancer cells

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Submitted by

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# Table of contents

# 1. Introduction

## 1.1 Colorectal cancer

Colon cancer (CC) and rectum cancer (RC) are uniformly named as colorectal cancer (CRC). These diseases are originated from the large bowel and sharing some pathomorphological and molecular features as well as similar risk factors.

### 1.1.1 CRC diagnosis

The signs and symptoms related to CRC diagnosis can be very diverse and non-specific. Patients with early-stage CRC are usually asymptomatic, and found through general screening. Obstructive symptoms and changes in bowel habits possibly occur in tumors of the transverse and descending colon. Bleeding is the most common symptom of RC [1, 2].

Colonoscopy is the first choice for the diagnosis of CRC. It is relatively easy to identify for advanced lesions, but not for early CRC. In order to ensure that these lesions are detected, complete mucosal examination and optimal bowel preparation are required [1, 3, 4].

Computed Tomography colonography is used as a complementary imaging method to diagnose polyps and CRC. However, imaging methods are mostly used for accurate local and distant stage [1, 5].

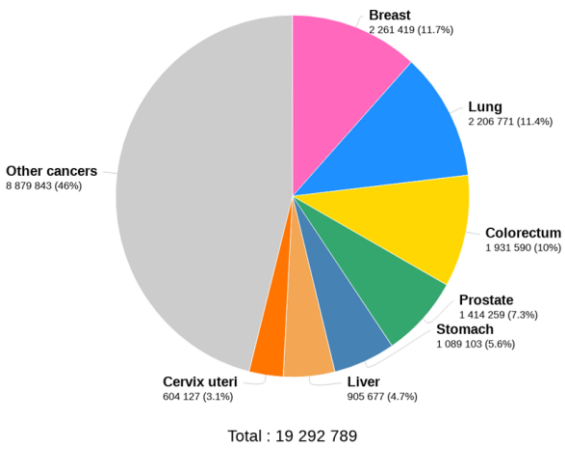
Pathology is still the basis of pathological staging and follow-up management. In addition to the classic TNM staging, histological classification, grading and histological evaluation of lymphatic, perineural and venous infiltration, histology also is used to check increasingly important tumor-markers such as mismatch repair tests and specific mutations [1, 6-8].

Moreover, laboratory analysis is also necessary; the guidelines recommend checking the concentration of carcinoembryonic antigen at the time of diagnosis [9]. Increased baseline carcinoembryonic antigen concentration is associated with poor prognosis, or failure of return to normal status within a period of time after surgery may indicate residual disease [1].

### 1.1.2 CRC epidemiology

In global cancer, the recent cancer statistics in 2020 presented that 1,931,590 individuals for both genders and all ages combined were diagnosed with CRC, and 935,173 patients with CRC died from this disease as the second common cancer-related deaths (Figure 1).

A

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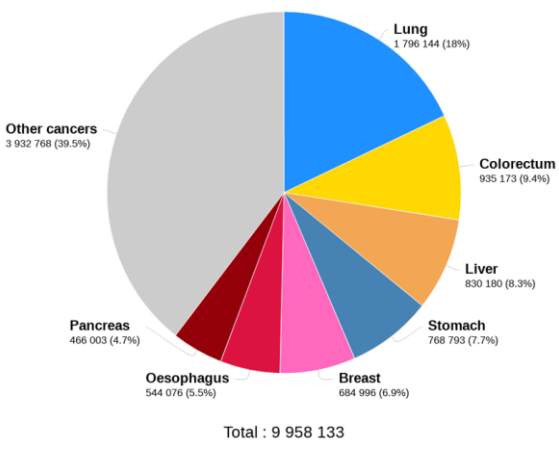


Figure 1. (A) Estimated number of new cancer cases for both sexes and all ages in 2020, worldwide; (B) Estimated number of deaths from cancers for both sexes and all ages in 2020, worldwide. Data source: Globocan 2020; Graph production: Global cancer observatory (http://gco.iarc.fr).

The 5-year prevalent cases of CRC were 5,253,335 with a proportion of 10.4% ranking second in all solid tumors. Among which Asia cases accounted approximately 50.0% (2,622,900 cases), followed by 29.2% Europe (1,536,168 cases) and 10.6% Northern America (554,680 cases) (Figure 2).

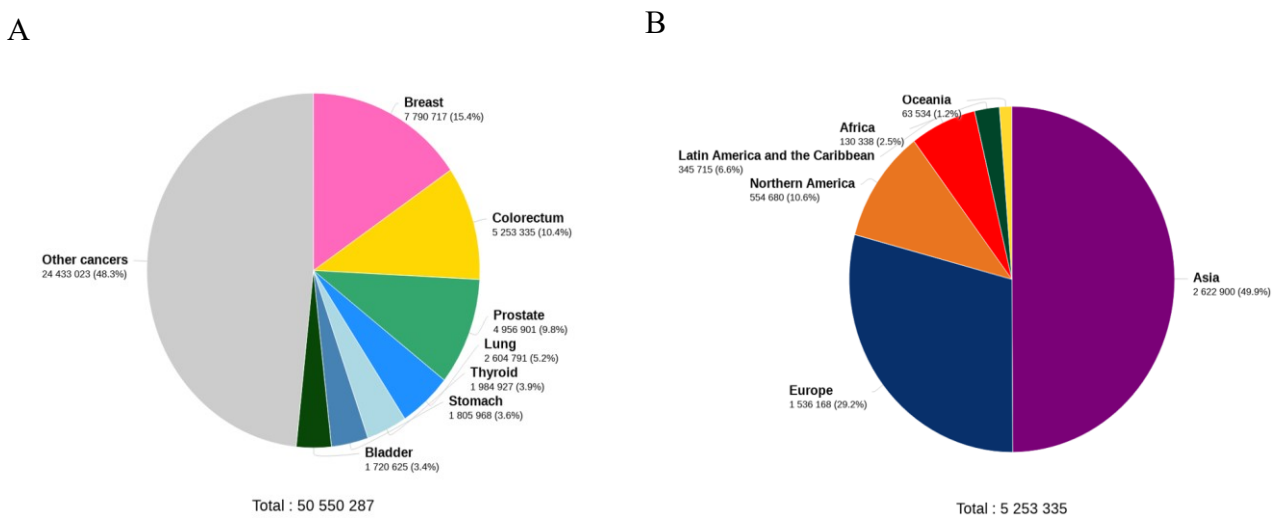



Figure 2. (A) Estimated number of 5-year prevalent cancer cases for both sexes and all ages in 2020, worldwide; (B) Estimated number of cases with CRC in different areas of world for both sexes and all ages in 2020. Data source: Globocan 2020; Graph production: Global cancer observatory (http://gco.iarc.fr).

In more detail, when describing at a national level, the global prevalence of CRC in 2020 was generally 67.4 per 100,000 with a range from 361.9/100,000 in Japan to 2.4/100,000 in Guinea. However, the highest prevalence of CRC was generally observed in Europe with the biggest rate of 205.1 per 100,000, Northern America secondly (150.4/100,000), closely followed by Oceania (148.9/100,000), then Asia (56.5/100,000), Latin America and the Caribbean (52.9/100,000) as well as Africa (9.7/100,000) (Figure 3). The burden in the prevalence of CRC in Western countries could be related to the genetic and environmental factors, especially dietary habits and lifestyle (alcohol

use, smoking as well as insufficient physical activity). In addition, a larger number of CRC occurred in Asia, to a large extent this phenomenon may possibly be attributable to the increasing trend of becoming westernized.

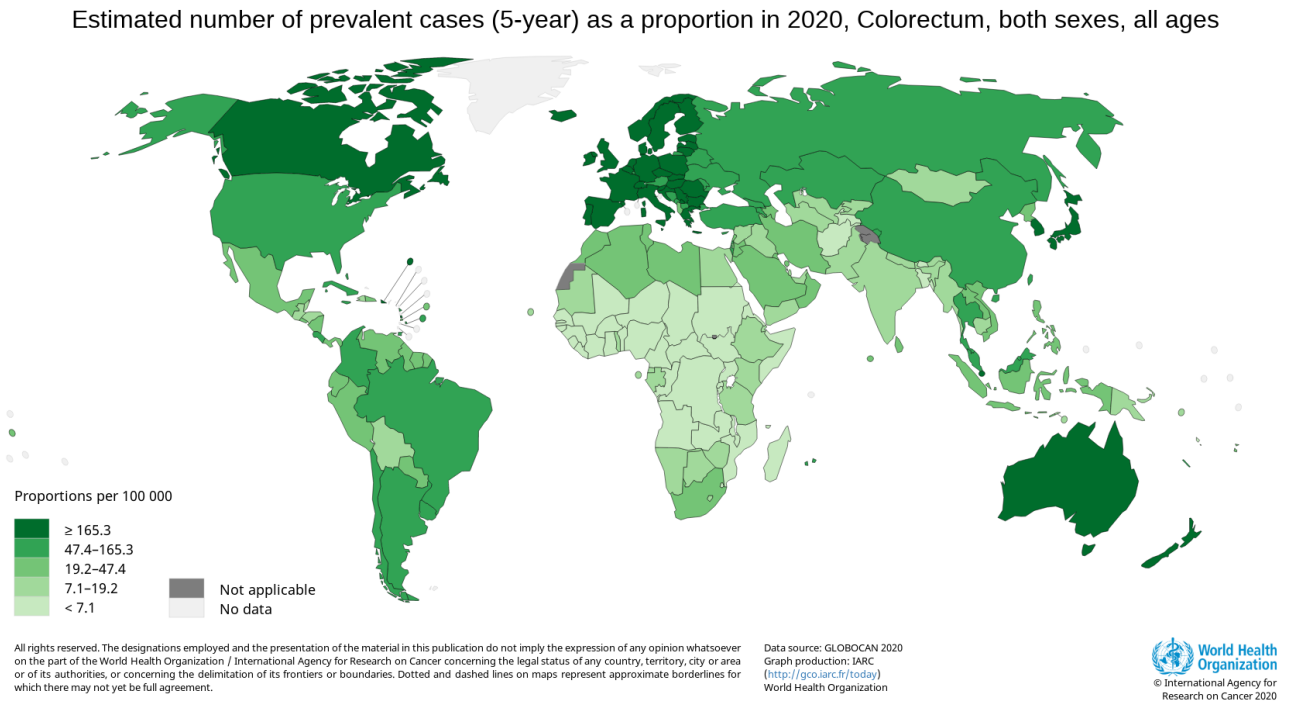



Figure 3. Estimated number of 5-year prevalent cases as a proportion in CRC for both sexes and all ages in 2020, worldwide; Data source: Globocan 2020; Graph production: Global cancer observatory (http://gco.iarc.fr).

### 1.1.3 Risk factors of CRC

The global burden of CRC is possible contributed to the following categories (Figure 4). Generally, the risk factors of CRC are classified as hereditary and environmental factors. Most environmental risk factors for CRC could be modified by changes in lifestyle, especially in diet. As reported [10-19], established risks for CRC include cigarette smoking, high intake of red, processed meat, obesity, lack of physical activity as well as multicausal combinations. On the contrary, diets rich in whole grains,

dairy products, tree nuts as well as foods containing dietary fiber, calcium and vitamin supplements have been found to protect from CRC.

Besides, based on the epidemiological statistics, the male gender or/and elders more frequently develop CRC [20]. Also, individuals with type 2 diabetes show an elevated risk of CRC development because of shared risk factors for both diabetes and CRC [21, 22].

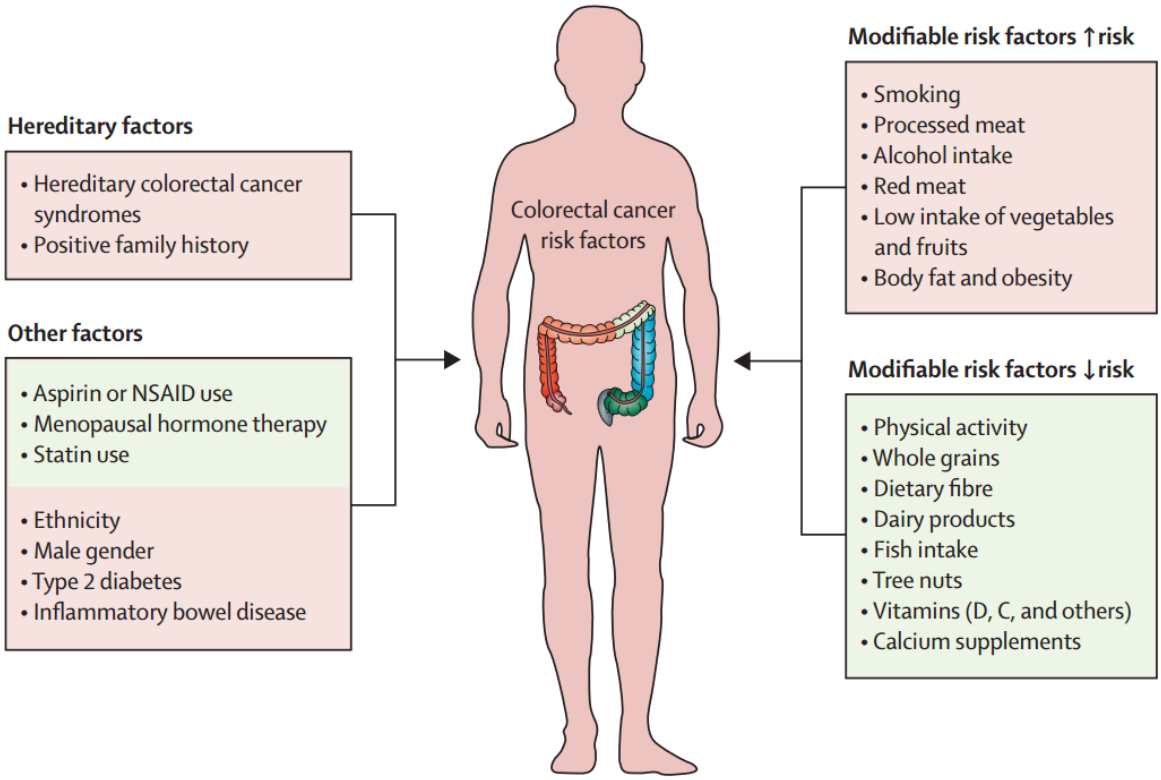



Figure 4. List of modifiable and non-modifiable risk factors for CRC [10]. NSAIDs: non-steroidal anti-inflammatory drugs.

On the other hand, genetic factors also play an important role in the development of CRC. The relative risk of CRC incidence varies from 0.89 to around 20-fold with the risk level from people without family history to people with inherited syndromes and a rising burden of family history. Moreover, CRC risk is associated with age of patient's at diagnosis: compared to people having the

same positive family history at an elder age, people with positive family history at a younger age have a higher relative risk of CRC development [10, 23]. However, most genetic factors are still elusive and need to be further studied [24].

Hereditary syndromes of CRC are clinically divided into polyposis syndromes and non-polyposis syndromes (lynch syndromes) based on presence or absence of numerous polyps (Figure 5).

Polyposis-related syndromes can be distinguished based on the histological type of polyps, their frequency of occurrence, the extent of extracolonic cancer as well as other clinical manifestations [25]. Patients with ten or more colon adenomatous polyps or two or more hamartomata's polyps usually are considered as hereditary polyposis syndrome and referral for genetic assessment is given. In addition, hereditary polyposis syndromes should be taken into diagnostic account for patients with polyps in other parts of the gastrointestinal tract, in younger individuals with a family history as well as patients with various gastrointestinal cancers. In general, combining comprehensive clinical evaluation, family history inquiry, polyp pathology and pathogenic gene germline testing could be used to determine this specific situation [26-28].

Lynch syndrome, an autosomal dominant genetic disease, is associated with a high risk of gastrointestinal cancer such as CRC, stomach cancer as well as small intestine cancer. Lynch syndrome is responsible for around 6% of CRC [29], is caused by germline mutations in the DNA mismatch repair (MMR) genes MutL homolog 1 (MLH1) [30], MutS homolog 2 (MSH2) [31], MutS homolog 6 (MSH6) [32], post meiotic segregation increased 2 [33] and the epithelial cell adhesion molecule [34]. These genes recognize and correct DNA mismatches during replication. Such

mismatches occur particularly in microsatellites, which is known as microsatellite instability (MSI). In terms of functionality, the loss of MMR genes will lead to an accumulation of unfixed errors. Subsequently, this leads to the formation of adenomas and cancer [35].

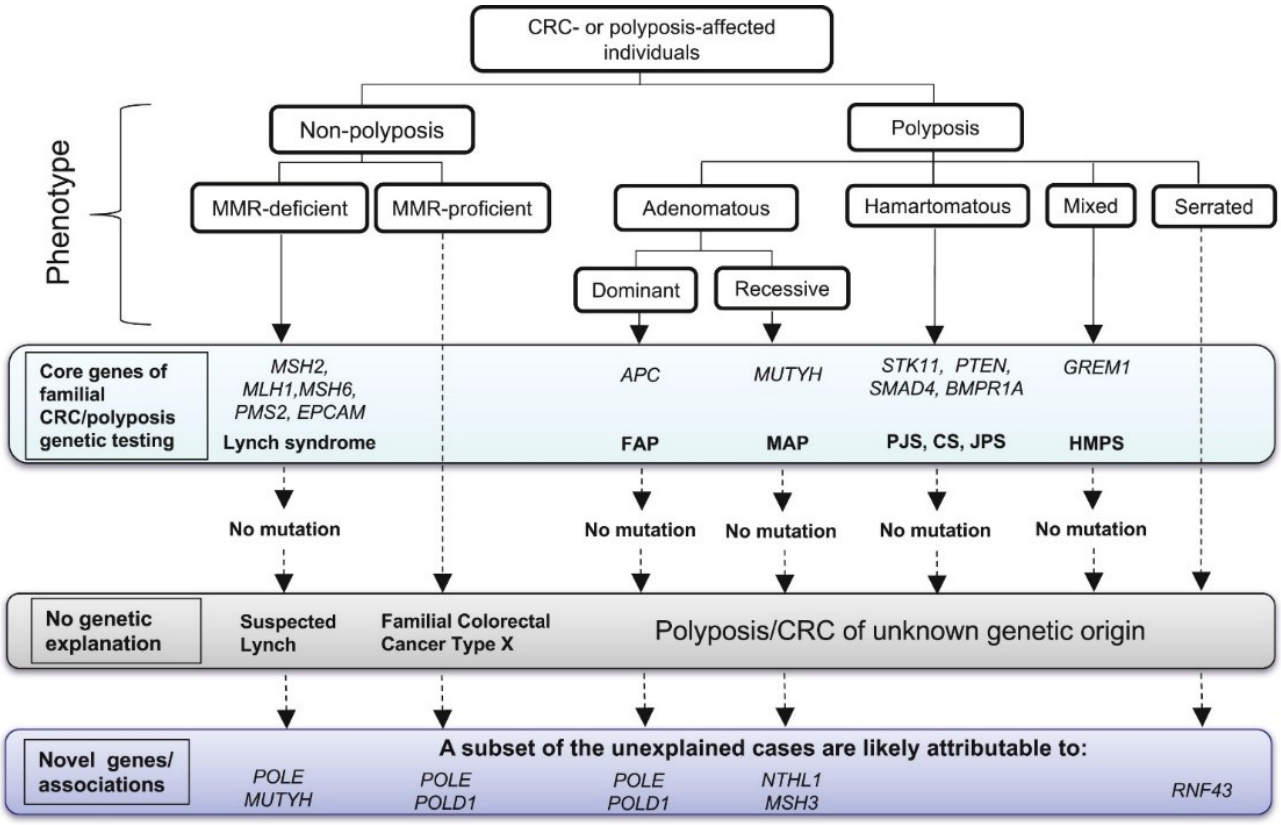



Figure 5. The flow chart of CRC in polyposis or non-polyposis syndromes describing the core gene of familial CRC and polyposis genetic testing (Bule box); the unclear genetic explanation for the significant proportion of the polyposis and non-polyposis associated CRC phenotypes (Gray box); novel genes identified (POLE, POLD1, NTHL1, MSH3, and RNF43) or CRC susceptibility genes related to differing phenotypes (MUTYH; Purple box). Dotted lines mean the cases without clear genetic origin. CS, Cowden syndrome; FAP, Familial adenomatous polyposis; HMPS, Hereditary mixed polyposis syndrome; JPS, Juvenile polyposis syndrome; MAP, MUTYH-associated polyposis; MMR, Mismatch repair; PJS, Peutz-Jeghers syndrome; FCCTX, Familial Colorectal Cancer Type X; MUTYH, MutY DNA glycosylase [25].

### 1.1.4 CRC progression

The development of CRC is related to several genetic and phenotypic changes in normal intestinal epithelial structure and function, leading to cell growth, proliferation as well as tumor progression. Since most CRCs are sporadic, rather than hereditary or familial, it is necessary to make efforts to explore these alterations, and to further improve the detection and treatments of CRC by identifying these changes. The sequence of adenoma-to-carcinoma during the tumor development reflects the accumulation of mutations in the colonic epithelium, which gives a selective growth advantage to the affected cells. These mutations occur at an early stage, usually in sporadic cases of adenoma and CRC. Somatic mutations must occur in multiple genes to form malignant transformation.

The process of transformation from adenoma to carcinoma is given in Figure 6. Briefly, the genetic model of colorectal tumorigenesis involved genomic instability, oncogene pathway activation, mutational inactivation or loss of tumor suppressor genes, and growth factor pathway activation [36].

Genomic instability includes three major molecular pathways which have been described for the progression of CRC: chromosomal instability (CIN) which causes changes in chromosome structure and copy number; MSI, which is initiated by aberrant DNA methylation or MMR gene mutations as well as CpG island methylator phenotype (CIMP) which is characterized by hypermethylation of a group of multiple genes [37-39].

Mutation or loss of APC, a tumor suppressor gene leads to the inappropriate activation of the Wnt signaling pathway, a mediator of the cell cycle, proliferation, and differentiation. In addition, mutational inactivation of TP53 causes loss of functions, such as G1 cell cycle arrest for facilitating

DNA repair, and failure of apoptosis induction. More seriously, inactivation of the transforming growth factor-β (TGF-β) signaling pathway could promote the adenoma transformation into a higher-grade dysplasia or carcinoma, and it also inactivates SMAD4. Besides, mutations of Ras gene family members (KRAS, HRAS, and NRAS) plus BRAF will stimulate cell proliferation and other activities that promote carcinogenesis by activating the mitogen-activated protein kinase (MAPK) signaling pathway [40, 41]. Moreover, mutations in PIK3CA and PTEN are related to disturbed PI3K signaling pathway, and also have an effect on cell growth, proliferation, and survival [42].

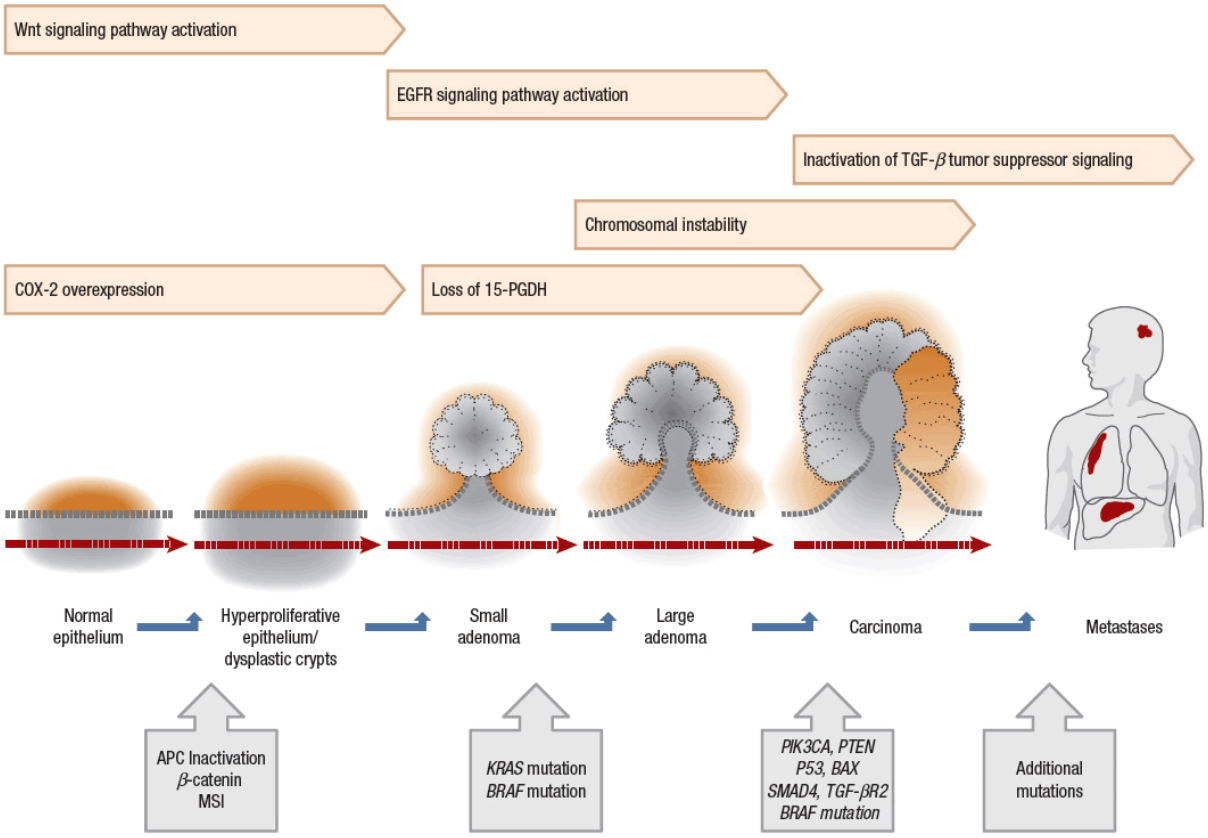



Figure 6. The genetic changes associated with progression of CRC from adenoma to carcinoma (https://basicmedicalkey.com/colorectal-cancer-2/).

The key growth factor pathways have been shown at the bottom of Figure 6. Activation of prostaglandin signaling is activated by the cyclooxygenase-2 (COX-2) overexpression in an early

step of the transformation process from adenoma to carcinoma. Further, most of CRC lost the 15-prostaglandin dehydrogenase (15-PGDH) which is responsible for degradation of prostaglandin. In addition, epidermal growth factor receptor (EGFR) is over-expressed or dysregulated in many human carcinomas including CRC [43], which influences cell growth, cell proliferation, differentiation, and angiogenesis. The activation of EGFR inhibits the mitogen-activated protein kinase (MAPK), PI3K as well as AKT signaling pathways, thus subsequently triggers colorectal tumorigenesis [36].

### 1.1.5 CRC prognosis

Nowadays, the entire prognosis of CRC patients has been improved by early detection as well as advances in therapeutic strategies [44]. Generally, the standard treatment for CRC includes surgery alone for patients with localized and low-risk regional tumor; surgery + adjuvant 5-FU-based chemotherapy for patients with high-risk regional tumor and surgery + a variety of chemotherapies + targeted treatment for patients with distant metastatic tumor. However, compared to the patients diagnosed in localized disease, those patients who have distant metastasis still show a very poor 5-year relative survival rate; around 90% vs. 14% [45].

The prognosis of CRC is based on a variety of factors, even the specific tumor stage is a very significant prognostic factor, but not sufficient to make an advanced treatment decision. Currently, the identification and definition of molecular and genetic markers let more patients with CRC benefit since it allows to choose an optimal therapy regimen for specific CRC subgroups. A majority of studies reported that patients with MSI-High or deficient MMR status have a significantly better

prognosis when compared to those with microsatellite stability (MSS) or proficient MMR, especially for patients diagnosed in stages II and III colon cancer [46-49]. However, these patients with MSI cancer in stage II or stage III failed to show a favorable outcome after adjuvant chemotherapy with fluorouracil-based regimens [48, 50, 51].

Moreover, a clinical trial for patients with stage III colon cancer, based on the detection of mutations in BRAF (V600E) or KRAS as well as MMR status, showed that patients with proficient MMR plus BRAF/KRAS mutations had significantly shorter survival outcome compared to those patients lacking these mutations [49]. Similar prognostic outcome was also observed in a majority of clinical trials [52-56]. These clinical trials suggested that MMR, BRAF and KRAS status could be considered as predictive genomic biomarkers for prognosis of CRC patients and subsequently taken into account for the guideline of the adjuvant setting [57].

APC is most frequently mutated in CRC, however, its prognostic and predictive ability remains inconsistent [57]. A retrospective study reported that status of APC did not affect survival outcome in CRC patients [58]. However, Wang et al. [59] reported that patients with APC wild type overall survive shorter than those with mutant APC in MSS metastatic CRC (mCRC), regardless of the RAS and BRAF status. On the contrary, liver mCRC patients with double mutations of APC and PIK3CA were associated with inferior response to preoperative chemotherapy and worse survival outcome when compared to patients without mutations [60].

The predictive ability of PIK3CA alone for prognosis is not clear, neither. Double mutations of PIK3CA and TP53 are associated with a poor prognosis for patients diagnosed in II and III stages

receiving 5-fluorouracil-based therapies [61]. Some studies reported that aspirin use could decrease the total mortality for CRC cases with mutant PIK3CA, but not for those with wild-type PIK3CA [62-64]. However, contrary to the original hypothesis, in a study using a large number of patients, it was observed that regular aspirin use doesn't significantly improve survival for PIK3CA-mutated CRC patients [65, 66].

Furthermore, conflicting results were obtained in several studies concerning the role of TP53 for survival prediction in CRC. Some studies reported that CRC patients with mutant TP53 have a poor survival outcome [67, 68] and seem not to benefit from chemotherapies [69]. However, Kandioler et al. [70] presented that mutant TP53 was significantly associated with a good prognosis in CRC patients with N1 treated by adjuvant 5-FU-based chemotherapy, whereas no prognostic significance was seen in CRC patients with N2 tumors. On the other hand, other studies found that TP53 mutations did not have an effect on survival outcome of CRC patients [58, 71-73]. Therefore, mutations of TP53, APC as well as PIK3CA, cannot be defined as prognostic and predictive biomarkers and need to be further explored in even larger cohorts of CRC patients.

## 1.2 Curcumin

Curcumin, a yellow coloring agent, is a phytochemical derived from Curcuma longa (turmeric) which belongs to the ginger family [74, 75] (Figure 7). In terms of chemical characteristics, the molecular formula of curcumin is $C_{21}H_{20}O_6$ , with a molecular weight of 368.4g/mol and a melting point of 183 °C. Curcumin is insoluble in water as a condition of acidic and neutral pH, but soluble in ketone, alkali, acetic acid, chloroform as well as dimethyl sulfoxide [74, 76].

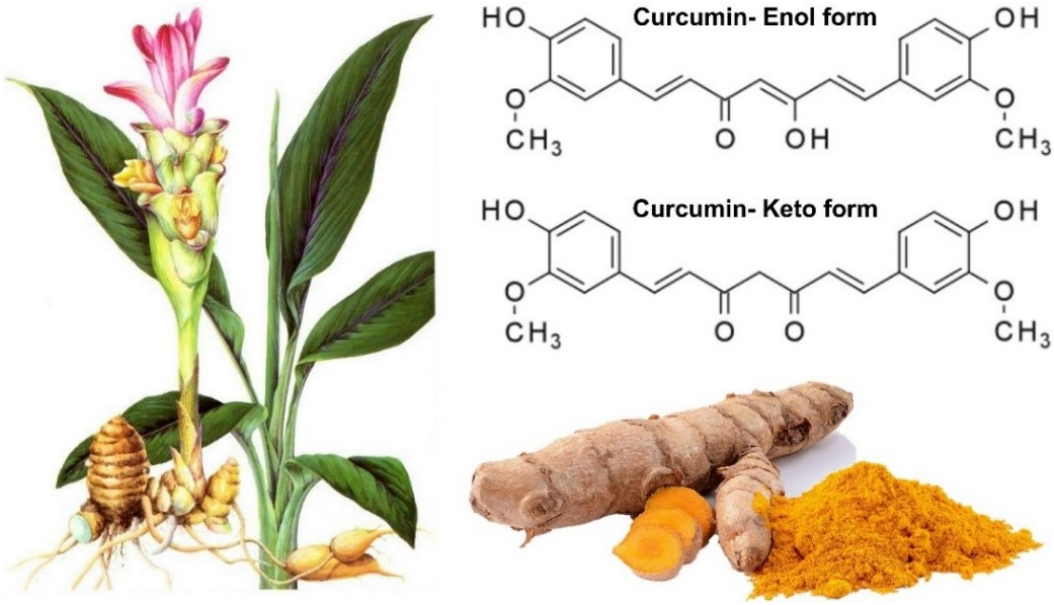



Figure 7. The molecular structure of curcumin isolated from the root of turmeric [75].

Curcumin was firstly isolated by Vogel in 1815 and its chemical structure was defined by V. Lampe and J. Milobedzka (Germany) in 1920. Commercially available curcumin also includes respectively 17% and 3% of demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) [76, 77].

Curcumin is usually utilized as an element of dietary supplements, a component of cosmetics, and a flavoring for foods and beverages especially in South and Southeast Asia [76]. Besides and more

importantly, curcumin also is widely applicated in the field of medicine. For example, traditional Chinese medicine with a long history of over 2000 years includes various forms of herbal medicine [78], curcumin as well as its active derivatives is one of the most important medicine and used as a novel therapeutic agents for multiple diseases, such as cardiovascular disease [78-81], metabolic disorders [82, 83], rheumatic autoimmune diseases [84], neurological disease [76, 85-87], skin diseases [88] [89] as well as neoplastic disease [90-92] (Figure 8).

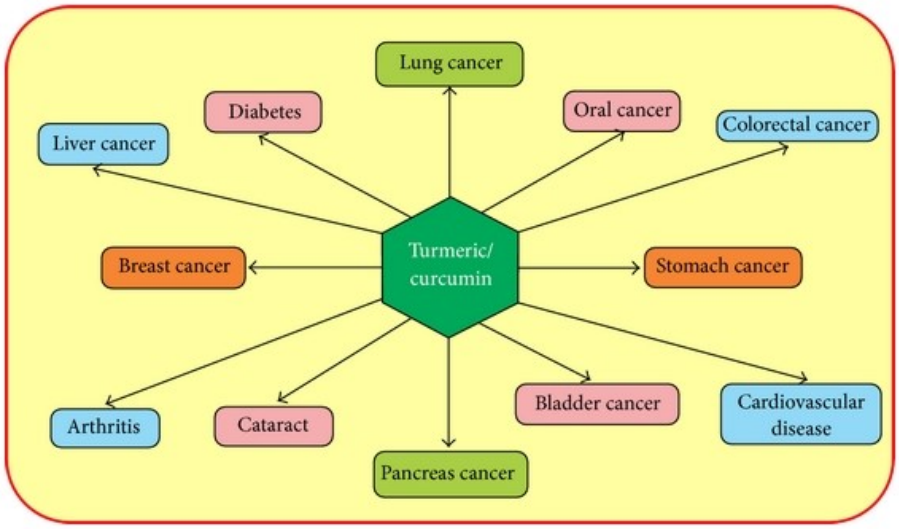



Figure 8. Turmeric/curcumin shows pivotal role in the prevention of diseases including cancer [93].

The majority of studies indicated that curcumin has potential in both prevention and treatment of cancers. Curcumin has been well shown to suppress the cellular transformation, initiation, promotion and/or progression, invasion and metastasis [94, 95]. The studies used orthotopic / xenotransplant models and employed curcumin alone / curcumin in combination with existing current therapies. In rodents, curcumin was used for the treatment of transplanted human cancers such as, prostate [96, 97], breast [98, 99], colon and rectum [100-102], ovary [103], lung [104], pancreas [105, 106], stomach [107], esophagus [108] and bladder [109].

## 1.3 CRC and curcumin

### 1.3.1 Functions *in vitro*

In summary, curcumin has been reported as an agent for CRC *in vitro*, which is able to inhibit growth [110-113], accelerate apoptosis [111, 113, 114], make cell cycle arrest in G2/M stage [112, 115] as well as suppress cell migration and invasion [110, 112, 116].

Curcumin was found to act on various signaling pathways as well as molecular targets, such as enzymes COX-2, transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and beta-catenin), Bcl-2 family members (Bcl-2, Bax, and Bcl-xL), death receptors (death receptor 5 (DR5) and Fas), protease enzymes (caspase 3 and caspase 8), and also reactive oxygen species (ROS) [100] (Figure 9).

In detail, compared to the paired adjacent normal colorectal mucosa, the COX-2 is over-expressed in most of CRC and it has even been suggested as a potential biomarker for CRC [117]. Moreover, several studies [116, 118] reported that patients with COX-2 positive CRC have a poorer prognosis, compared to those with COX-2 negative CRC. However, Zhou et al. [119] and Kunnumakkara et al. [113] found that curcumin has an effect on the antitumor and anti-metastatic activity by inhibiting NF-κB activation itself as well as NF-κB-regulated gene expression including cyclin D1, Bcl-2, Bcl-xL, COX-2 and MMP-9. In addition, Chen et al. [120] reported that curcumin inhibited the growth of CC cells by suppressing EGFR expression through decreasing the trans-activation activity

of Early growth response protein 1 (Egr-1). On the other hand, curcumin can increase TP53 expression and consequently speed up the apoptosis of CRC [114, 121] (Figure 9).

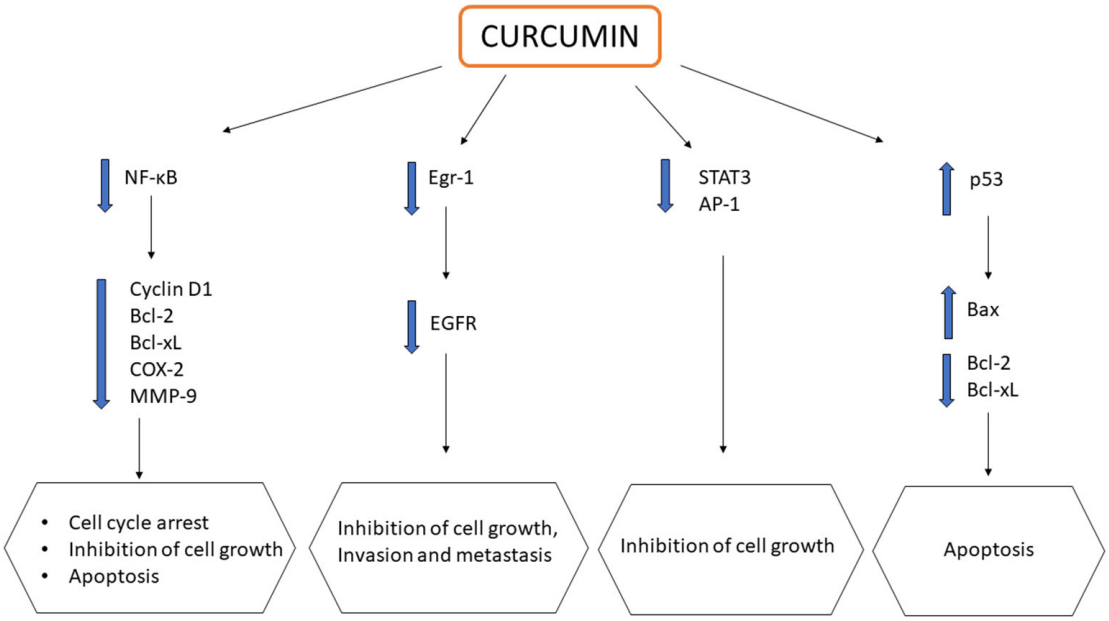



Figure 9. Schematic representation of curcumin impact on multiple signaling pathways in cancers [122].

In fact, most of the anti-inflammatory and -tumor properties of curcumin are considered to be mainly block the NF-κB pathway activation, which has been investigated to be up-regulated in CRC representing a major cause for drug resistance [123, 124].

At this time, chemotherapy is regarded as the basis of treatment and improves the prognosis for mCRC by multiple iterations of two or three chemo-drugs regimens. However, development of drug resistance is still a remarkable challenge [125-127], hence the exploration for more effective drugs always needs to continue. Porras et al. [128] reported that hyper-activated NF-κB in CRC cell lines is associated with resistance to oxaliplatin, and further determined the synergistic effect of oxaliplatin and curcumin in cell lines with acquired resistance to oxaliplatin. The most possible explanation is that curcumin inhibits the NF-κB signaling cascade, accordingly causing a reversion of the resistant

phenotype [128]. This phenomenon was continuously seen in the combination of conventional 5-FU and curcumin which led to more therapy effects against chemo-resistant CRC cells. It might also involve down-regulated NF-κB gene products via the NF-κB/PI-3K/Src pathway [129]. Moreover, curcumin was also found to block radiation-induced NF-κB activation thereby potentiating the antitumor effects of radiation therapy [130].

### 1.3.2 Functions *in vivo* and clinical trials

In an animal model, Perkins et al., [131] in 2002, demonstrated that an intake of curcumin at 0.2%, which corresponded to 300mg/kg, prevented or delayed adenoma development. In detail, this study used C57BL/6J Min/+ (ApcMin/+) mice as a model for human familial adenomatous polyposis. These mice received a standard diet with curcumin concentrations of 0.1%, 0.2% and 0.5% for 15 weeks. The 0.1% group did not show any effect, whereas the higher concentrations of 0.2% and 0.5% reduced the intestinal tumor number significantly by 39% and 40%, respectively [131].

A phase I clinical trial [132] included 15 advanced CRC cases who were resistant to standard chemotherapies. Oral curcumin was used to replace the classical chemo-drugs by consuming capsules with curcumin leading to doses between 0.45 and 3.6g daily for up to 4 months. Dose-limiting toxicity was not observed. Curcumin and its glucuronic acid and sulfate metabolites were detected in plasma and urine at a concentration of 10nmol/L. PGE(2) was used as a biomarker of the potential activity of curcumin and measured in blood leukocytes of patients on days 1 and 29. The results showed that daily dosing of curcumin at 3.6g engendered 62% and 57% decreases in inducible PGE(2) in blood samples taken 1 hour after intake, respectively, compared with levels

observed immediately pre-dose [132]. However, an early clinical study using human volunteers reported that the curcumin serum concentration 1 hour post-administration of 2g curcumin alone was very low (less than10 ng/ml), even undetectable [133]. Moreover, Wong et al. [134] reported that the serum curcumin concentration is only in the nanomolar range, indicating poor absorption and low bioavailability; consequently limiting its clinical potential.

### 1.3.3 Development of curcumin nano-formulations and novel formulations

As a result, this issue gives impetus to the development of curcumin nano-formulations and novel curcumin formulations in the quest of improving curcumin bioavailability in cancer. An example is Meriva®, a novel curcumin formulation, which displayed clear efficacy in a xenograft model. The combination of Meriva® plus oxaliplatin was better than Meriva® alone, followed by oxaliplatin and the control. The decrease in tumor volume when compared to vehicle-treated animals was 53%, 35% and 16%, respectively for the treatments. Addition of formulated curcumin to oxaliplatin-based chemotherapy regimens has the potential for clinical benefit [135]. Some clinical trials using novel curcumin formulations are ongoing with the goal to evaluate safety and efficacy in CRC prevention and treatment (shown in Table 1).

Moreover, co-administration of 2g curcumin and 20mg piperine resulted in a remarkable 2,000% increase in oral bioavailability. It means that piperine has the ability to alter the metabolism of curcumin by blocking the glucuronidation process in the liver and intestine, therewithal significantly improve the curcumin oral bioavailability [133]. Besides, other promising oral drug delivery systems that can increase the absorption rate up to 100 times have been developed, including liposomes,

micelles, phospholipid complexes, microemulsions, nanoemulsions, emulsions, solid lipid nanoparticles, nano structured lipid carriers, biopolymer nanoparticles and microgels [123, 136, 137].

Table 1. Summary of ongoing clinical trials with bioavailable curcumin formulations in CRC [123].

| Study Title | Condition | Intervention | Primary porpouse | Enrollment | Trial Phase | Allocation | Current Status | NCT Number |
|---|---|---|---|---|---|---|---|---|
| 1. First Line Avastin/ FOLFIRI in Combination with Curcumin containing Supplement in Colorectal Cancer Patients with Unresectable Metastasis | CRC patients with unresectable metastasis | **Drug**: Avastin/FOLFIRI **Dietary Supplement**: Nanostructured lipid curcumin particles (100 mg) | PFS | 50 | 2 | N/A | Completed | NCT02439385 |
| 2. A Prospective Evaluation of the Effect of Curcumin on Dose-limiting Toxicity and Pharmacokinetics of Irinotecan in Colorectal Cancer Patients | Advanced CRC patients | **Drug**: Irinotecan **Dietary Supplement**: Meriva® (curcumin in a matrix of microcrystalline cellulose combined with soy lecithin Phosphatidylcholine) | PKMTD | 23 | 1 | Non-Randomized | Active, not recruiting | NCT01859858 |
| 3. A Pilot, Feasibility Study of Curcumin in Combination With 5FU for Patients With 5FU-Resistant Metastatic Colon Cancer | 5FU-Resistant Metastatic CRC patients | **Drug**: 5-flurorouracil **Drug**: BCM-95: micronized rhizome extract containing phospholipids and 500 mg of pure curcuminoids (95% curcumin, 5% desmethoxycurcumin) suspended in turmeric essential oil | Safety | 13 | 1 | N/A | Active, not recruiting | NCT02724202 |
| 4. Phase I Clinical Trial Investigating the Ability of Plant Exosomes to Deliver Curcumin to Normal and Malignant Colon Tissue | CRC patients | **Dietary Supplement**: Curcumin **Dietary Supplement**: Curcumin conjugated with plant exosomes **Other**: No Intervention | Curcumin concentration in normal and cancerous tissue | 7 | 1 | Randomized | Active, not recruiting | NCT01294072 |
| 5. Phase I Pharmacokinetic Trial of Curcuminoids Administered in a Capsule Formulation | CRC patients | **Dietary Supplement**: Curcuminoids in capsule formation | Safety PK MDT | N/A | 1 | N/A | Completed | NCT00027495 |
| 6. Randomized Window of Opportunity Trial of Anthocyanin Extract and Phospholipid Curcumin in Subjects With Colorectal Adenoma | Patients with colorectal adenoma | **Dietary Supplement**: Mirtoselect® + Meriva® **Dietary Supplement**: Placebo | Change of IHQ expression of β-catenin in normal and adeno colon tissue | 100 | N/A | Randomized | Active, not recruiting | NCT01948661 |

In summary, these data suggest that therapeutic curcumin use seems to be safe in humans, thus the design and development of new clinical trials focused on novel curcumin formulations as well as curcumin nano-formulations should be encouraged to clarify its clinical efficacy.

## 1.4 Aim of the study

Improving clinical application should not only focus on curcumin bioavailability, but also on the identification of specific CRC subsets with best sensitivity to curcumin.

It is also noteworthy to mention, that different CRC cell lines responded to treatments differently, possibly due to genomic instability or phenotypic changes [122]. I hypothesized that also the sensitivity to curcumin might be linked to specific molecular features such as MSI status, mutations or certain activated molecular signaling pathways.

Therefore, the present study aimed at 1) testing the sensitivity of CRC cell lines with defined molecular alterations to curcumin; 2) mechanistically analyzing sensitive CRC cell lines in order to identify the mode-of-action; 3) identifying biomarkers associated with curcumin sensitivity; 4) establishing a predictive model using biomarkers to predict the sensitivity of additional CRC cell line to curcumin.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Instruments

| **Instruments** | **Manufacturers** |
|---|---|
| BD FACS Calibur | Becton Dickinson, Heidelberg, Germany |
| Centrifuge 5415 D | Eppendorf, Hamburg, Germany |
| Centrifuge Rotina 420 R | Hettich, Tuttlingen, Germany |
| Freezer (-20°C) | Bosch and Kryotech, Germany |
| Freezer (-80°C) | Bosch and Kryotech, Germany |
| Fridge (4°C) | Liebherr, Germany |
| Heater HBT-1-131 | Heap Labor Consult, Bovenden, Germany |
| Incubator | Thermo Fisher Scientific, Waltham, USA |
| Light microscope CKX 41 | Olympus Deutschland, Germany |
| Tecan Infinite M200 Microplate Reader | TECAN, Hudson, USA |
| Cellular Technology Ltd. ELISpot reader | ImmunoSpot, USA |
| Neubauer-counting chamber | Marienfeld, Lauda Konigshofen, Germany |
| Pipetboy | Integra Bioscidence, Fernwald, Germany |
| Vortex Genie 2 | Scientific Industries, New York, USA |
| MSC-Advantage Class II Biological safety cabinets | Thermo Fisher Scientific, Waltham, USA |
| IKA digital 3D rocker | Thermo Fisher Scientific, Waltham, USA |
| Multi-stepper | Eppendorf, Hamburg, Germany |
| Brand Handystep | Sigma, Germany |
| Pipettes | Eppendorf, Hamburg, Germany |

### 2.1.2 Disposable products

| Disposable Products | Manufacturers |
|---|---|
| Cell culture flasks | Greiner Bio-One, Kremsmünster, Austria |
| Cell culture plates | Greiner Bio-One, Kremsmünster, Austria |
| Falcon tubes cellstar | Greiner Bio-One, Kremsmünster, Austria |
| FACS tubes | Sarstedt, Nümbrechus, Germany |
| Micro tubes | Sarstedt, Nümbrechus, Germany |
| Pipette tips | Eppendorf, Germany |
| Serological pipettes | Greiner Bio-One, Kremsmünster, Austria |
| Pasteur pipettes | Thermo Fisher Scientific, Waltham, USA |
| Dispenser tips | TipOne, Germany |

### 2.1.3 Chemicals and Reagents

| Chemicals and Reagents | Manufacturer |
|---|---|
| Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 | Pan-Biotech, Aidenbach, Germany |
| DMEM/Ham's F12 | Capricorn Scientific GmbH, Germany |
| L-glutamine | Pan-Biotech, Aidenbach, Germany |
| Dulbecco's phosphate buffered saline (DPBS) | Pan-Biotech, Aidenbach, Germany |
| Fetal bovine serum | Pan-Biotech, Aidenbach, Germany |
| Trypsin/EDTA | Pan-Biotech, Aidenbach, Germany |
| 70% Ethanol | Apomix, Germany |
| Fetal bovine serum | Pan-Biotech, Aidenbach, Germany |
| 4% Formafix | Formafix GmbH, Düsseldrof, Germany |
| Propidium iodide (PI) | AppliChem, Darmstadt, Germany |
| Trypan blue (0.02%) | Sigma-Aldrich, Hamburg, Germany |

| | |
|---|---|
| Dimethyl sulfoxide (DMSO) | AppliChem, Darmstadt, Germany |
| Sodium chloride | Carl Roth GmbH, Germany |
| Potassium chloride | MERCK, Germany |
| Di-sodium hydrogen phosphate dihydrate | MERCK, Germany |
| Potassium di-hydrogen phosphate | MERCK, Germany |
| Saponin | AppliChem, Darmstadt, Germany |
| Ethanol absolute | Walter CMP, Kiel Germany |
| Sodiumdodecylsulfat (SDS) | AppliChem, Darmstadt, Germany |
| Natrum chorid (NaCL) | Roth, Germany |
| Tween 20 | Sigma-Aldrich, Hamburg, Germany |
| RNase | Sigma-Aldrich, Hamburg, Germany |
| HEPES | Sigma-Aldrich, Germany |
| PE anti-IκB-α | BioLegend, Germany |
| Annexin V | Immuno Tools, Germany |
| Curcumin | Sigma-Aldrich, Germany |

### 2.1.4 Buffer and Medium

| **Buffer and Medium** | **Components** |
|---|---|
| Cell culture medium | 445ml DMEM/Ham's F12 medium; 50ml FBS; 5ml L-glutamine |
| Freezing medium | 45ml serum; 5ml DMSO |
| 10 × PBS | 80g sodium chloride; 2g potassium chloride; 18.05g di-sodium hydrogen phosphate dihydrate; 2g potassium di-hydrogen phosphate; 1000ml $dH_2O$; pH: 7.2 - 7.4 |
| 100 × Buffer P | 0.5ml FBS; 5ml 1% Saponin; 5ml 0.1mol/l HEPES; 39.5ml 1 × PBS |

| | |
|---|---|
| 10 × Binding buffer | 0.1mol/l HEPES; 1.4mol/l NaCl; 25mmol/l $CaCl_2$•2H2O in distilled water |
| Crystal violet (Final: 0.2%) | 200mg crystal violet; 2ml ethanol absolute (final 2%); ad 100ml distilled water |
| Sodiumdodecylsulfat (SDS, Final: 1%) | 1g SDS; ad 100ml distilled water |

### 2.1.5 Patient consent

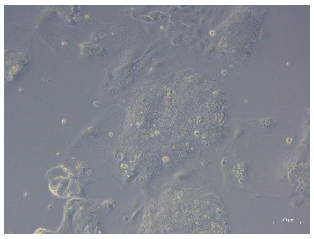
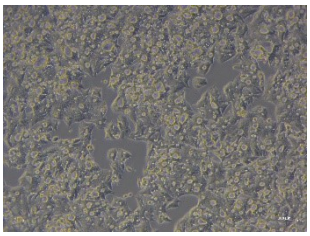
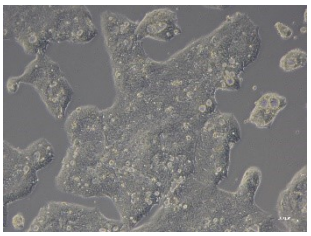
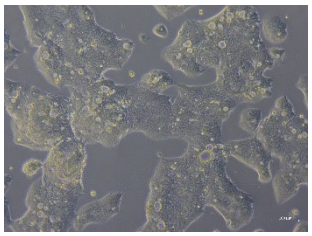
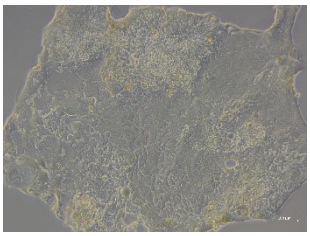
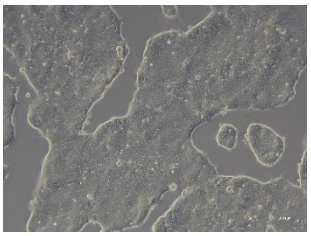
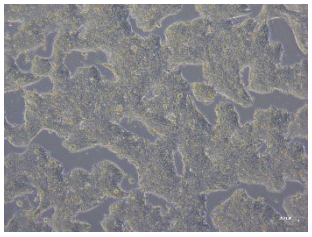
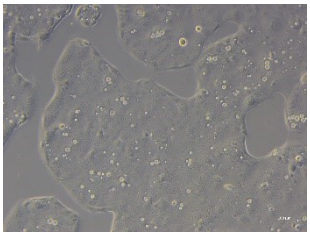
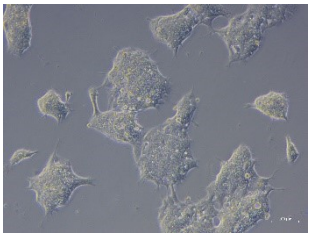
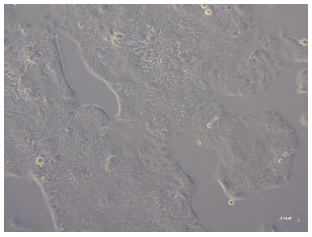
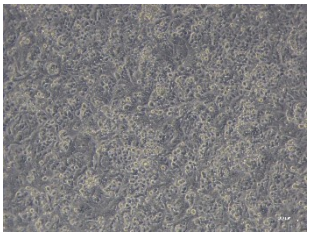
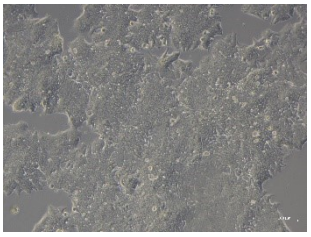
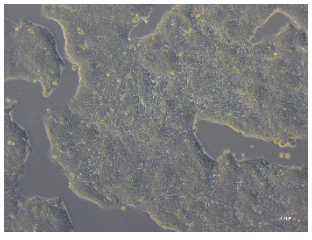
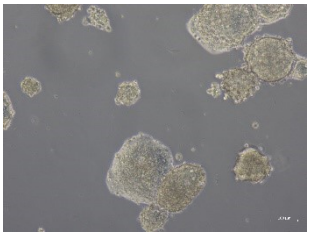
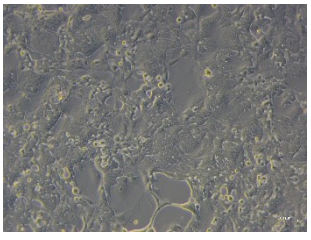
After patient consent obtained, the primary samples were collected from the surgery. The further research was approved by the Ethics Committee of the University of Rostock (Reference number: II HV 43/2004) based on guidelines for the use of human material. The present study included age, sex (male, female), tumor location (colon: right, transverse, left, sigmoid and rectum), T stage (T0, T1, T2, T3 and T4), N stage (N0, N1and N2), M stage (M0 and M1), UICC stage (I, II, III and IV), grade (G1, G2, G3 and G4), mutations (APC, TP53, KRAS, BRAF, PIK3CA and ATM) and MSI status (MSI and MSS).

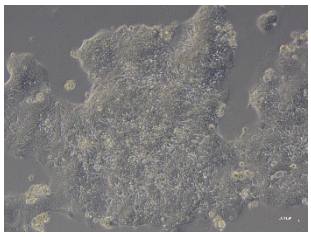
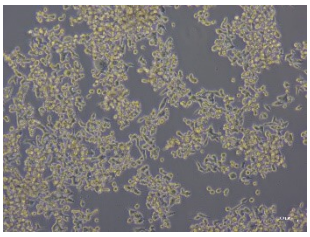
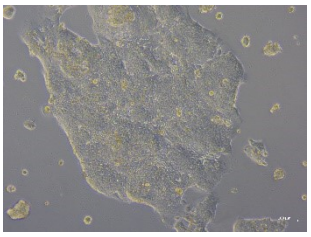
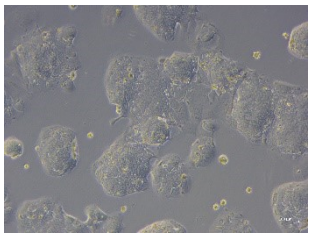
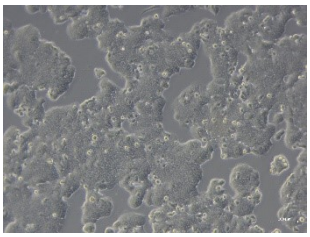
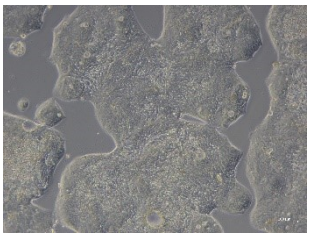
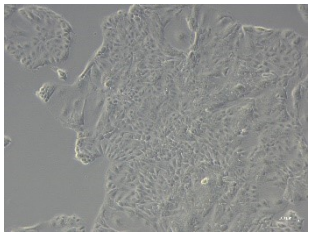
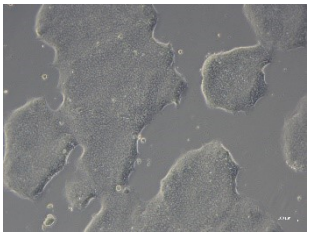
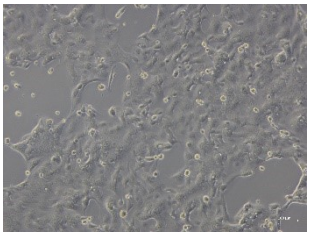
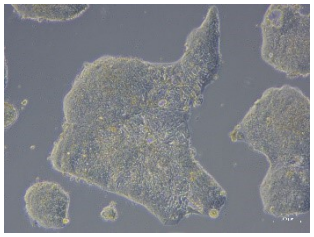
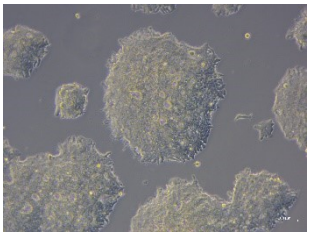
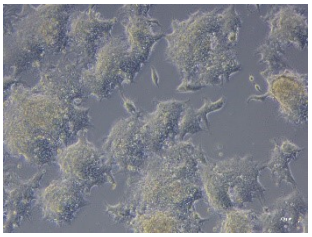
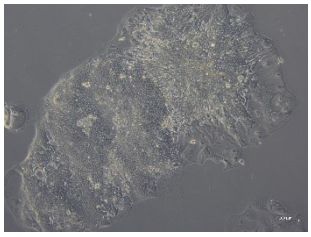
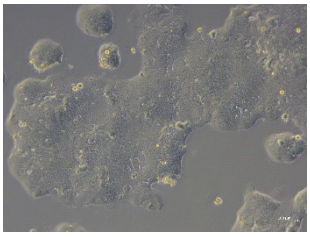
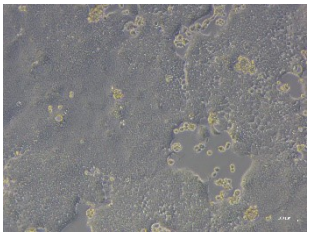
### 2.1.6 CRC cell lines

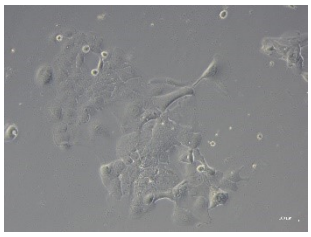
A large panel of low passage cell lines has been established in Molecular Oncology and Immunotherapy group, included CRC patient-derived / PDX-derived as well as primary / metastatic cell lines [138].

Finally, 32 CRC cell lines were used in present study including 31 HROC cell lines and HHC6548.

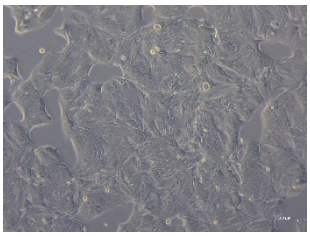
List of CRC cell lines in this cohort was shown in followed pictures.

| | | |
|---|---|---|
|  HROC18; T2N0M0; G2; UICC I |  HROC24; T2N0M0; G2; UICC I |  HROC32; T4N2M1; G2; UICC IV |
|  HROC39; T4N0M0; G3; UICC II |  HROC43; T3N2M0; G3; UICC III |  HROC50 T1 M5 (HROC50); T4N0M0; G2; UICC II |
|  HROC57; T3N2M1; G3; UICC IV |  HROC60; T2N0M0; G2; UICC I |  HROC69; T3N0M0; G3; UICC II |
|  HROC80 T1 M1; T3N2M0; G2; UICC III |  HROC87 T0 M2 (HROC87); T3N0M0; G3; UICC II |  HROC113; T4bN2M0; G3; UICC III |
|  HROC126; T3N1M0; G2; UICC III |  HROC131; T3N1M0; G3; UICC III |  HROC173; T4N2M1; G3; UICC IV |

| | | |
|---|---|---|
|  HROC183; T3N2M0; G3; UICC III |  HROC212; T4N2M1b; G3; UICC IV |  HROC217; T3N1M0; G2; UICC III |
|  HROC222; T3N0M0; G2; UICC II |  HROC257; T4N2M0; G3; UICC III |  HROC277 T0 M1 (HROC277); T4N0M1; G2: UICC IV |
|  HROC285 T0 M2 (HROC285); T4bN2M1; G2; UICC IV |  HROC296; T3N0M0; G2; UICC II |  HROC300 T2 M1 (HROC300); T4N1M1; G2; UICC IV |
|  HROC309; T3N0M0; G2; UICC II |  HROC315 T1 M2 (HROC315); T3N2bM0; G3; UICC III |  HROC324; T3N2M1a; G3; UICC IV |
|  HROC348; T3N2M1; G3; UICC IV |  HROC357; T3N0M0; G2; UICC II |  HROC370; T2N0M0; G2; UICC I |



HROC383; T3N0M0; G3; UICC I



HHC 6548 T1 M1 (HHC6548); T3N2Mx; G3; unclear UICC

CRC patient-derived cell lines are named by starting with HROC (Hansestadt Rostock colorectal cancer) and patient ID number, for example HROC24; PDX-derived cell lines were additionally plus the number of passage *in vivo* (shown as Transfer: T) and the number of mice (shown as M), for example HROC24 T0 M1; besides, the mCRC cell lines both patient- and PDX-derived plus the "Met" closed by the patient ID number, such as HROC277Met and HROC277Met T0 M2 [138].

These well-characterized and low-passage cell lines are comprehensive in comparison to the parental tumor by the morphology, phenotype, molecular characteristics, chemotherapeutic- and radiation-response. This biobank of CRC cells is considered as a useful tool for the future research on the biological characteristics and clinical studies [139-143].

## 2.2 Methods

### 2.2.1 Establishment of patient derived- or PDX derived- cell lines

In brief, the fresh tissues of CRC were obtained from clinical operation, immediately, these tissues were minced by the crossed scalpels in PBS, then passed through a cell strainer to collect some single cells. After washing using PBS, these cell pellets were seeded on 6-well plates with collagen-coated [138].

### 2.2.2 Mutation and molecular subtype of CRC cell lines

The information of mutations and MSI status has been previously performed. As described before, the mutational status was determined by testing of KRAS exon 2-4, BRAF exon 15, TP53 exon 5-8, APC exon 16, PIK3CA exon 10 and 21 [138, 144]. Molecular subtype was identified based on the research from Ostwald and colleagues on an ABI 3500 genetic analyzer (Applied biosystems, Darmstadt, Germany) [143-145].

### 2.2.3 Cell culture

Cell culture was done under a sterile laminar flow hood. The CRC cell lines were quickly thawed, after a centrifugation at 1200rpm for 10 minutes, resuspended in 5ml medium containing 10% FCS and 2mM L-glutamine and cultured in the T25 culture flasks. The medium was changed every 3 - 4 days. When the cells expand to an 80% confluence, subsequently, enzymatic cell detachment was

treated by trypsin. After an incubation time of 5 - 10 minutes at 37°C, the cell pellets were resuspended in a bigger T75 culture flask and grow in a humidified incubator (5% $CO_2$ and 37°C) for the further research.

The CRC cell lines lower than 50 passages were used in order to ensure the comparability of molecular characteristics between parental tumor and tumor-derived cell lines.

Moreover, to avoid contamination, mycoplasma test was performed every 6 weeks in routine by a 16S-rRNA gene-based PCR amplification from whole cell lysates. All cells used in present study showed negative results.

### 2.2.4 Cell counting

Cell counting was performed by trypan blue assay with the support of counting chamber. The trypan blue can discriminate the living cells from the dead cells by non-staining the nuclei of living cells. In present analysis, the mixture of 5µl cell suspension and 5µl trypan blue was loaded into the counting chamber. The cells from two squares of chamber were counted and calculated by this formulation: cell number / ml = (number of viable cells / numbers of the counted squares) × 2 × $10^4$.

### 2.2.5 Chemo-test and crystal violet

$1.0 \times 10^4$ - $1.5 \times 10^4$ CRC cells per well were seeded in a 96-well plate, and allowed to adhere for 24 hours. Cultures were treated by changing the medium with the increasing concentrations of curcumin

(0µM, 1µM, 5µM, 10µM, 15µM, 20µM and 25µM) and incubated for 72 hours. Then, medium containing curcumin was changed and left for incubation of another 72 hours.

After that, these cells were stained using crystal violet assay. In short, these cells after treatments were firstly washed using 100µl PBS, then added 50µl crystal violet solution per well, and incubated on a shaker for 15 minutes at room temperature. After that, the cells were washed twice, again. Photos were taken using the ELISpot reader after the cells were completely dried. These cells were quantified by adding 100µl 1% SDS per well and incubated on a shaker for 10 minutes at room temperature. Then the cells were measured by using the Tecan reader with an absorption at 570nm (reference: 620nm). Cell viability is calculated by the formula: cell viability = (sample – dead control) / (living control – dead control) × 100%.

### 2.2.6 Annexin V and propidium iodide staining

$2 \times 10^5$ CRC cells per well were harvested, put in a 6-well plate, and left for incubation overnight. These cells were treated by 5%FCS medium containing 0µM, 10µM, 15µM and 20µM curcumin for incubation of 72 hours. The cell death was evaluated by apoptosis assay with Annexin-V (Immuno Tools, Germany, cat: 31490013) and propidium iodide (PI, AppliChem, Germany, code: A2261) labeling. In brief, the cells were harvested and transferred into FACS tubes, washed by 500µl 1 × binding buffer. Cells were resuspended in 70µl 1 × binding buffer plus 5µl Annexin-V-FITC / non-Annexin-V-FITC by protected from light and incubated for 15 minutes at room temperature. After that, 400µl 1 × binding buffer was added in each tube. Finally, PI was just added within 1 minute before measurement.

The cell death was determined by the formula: 100% - (Annexin $V^-$ $PI^-$) %.

### 2.2.7 Evaluation of inhibitors of NF-kappa-B (IκB) level in CRC cell lines

$5 \times 10^5$ CRC cells per well were seeded in a 6-well plate for incubation of 24 hours; then changed the medium and allowed cells to adhere for 24 hours.

After that, the cell pellets were harvested and fixed using 400µl 2% FORMAFIX per tube for 15 minutes at room temperature; washed by 400µl PBS followed by an incubation using 400µl 1 × buffer P for 10 minutes at room temperature. Next, the cell pellets were resuspended in 100µl 1 × buffer P with and without 5µl anti-IκB-α (BioLegend, Germany, Cat: 662412) by protected from light and incubated for 30 minutes at 4°C. The cell pellets per tube were washed again by 400µl 1 × buffer P and resuspended using 200µl 2% FORMAFIX per tube, then the IκB expression was measured by flow cytometry.

### 2.2.8 Evaluation of IκB level in CRC cell lines after curcumin treatment

$5 \times 10^5$ CRC cells per well were seeded in a 6-well plate and left for incubation overnight; then changed medium by 5% FCS medium and 5% FCS medium containing 10µM, 15µM, 20µM curcumin and incubated of 24 hours. The procedure for measurement of IκB expression in pre-treated cells is same as evaluation of IκB level in CRC cell lines.

### 2.2.9 Cell cycle

Curcumin pre-treated CRC cells were harvested, followed by a wash step using 2ml PBS, then resuspended in 1ml ice-cold 70% ethanol, vortexed, added 1ml ice-cold 70% ethanol again for more than 12 hours incubation at -20℃. After that cell pellets were washed again using 2ml PBS, resuspended in 300µl PBS containing 0.1% Tween 20 and 1mg/ml RNase and incubated at 4℃ for 1 hour. The cell cycle was measured using the Cellquest program after adding 50µl 0.1mg/ml PI solution.

### 2.2.10 Wound healing assay

$5 \times 10^5$ cells per well were seeded in a 6-well plate and incubated until reaching 100% confluency. Then the medium was changed by DMEM/Ham's F12 medium or containing 10µM curcumin, and incubated for 24 hours in a humidified incubator. Afterwards, the monolayers were scratched using a 20µl pipette tip to generate a wound. Wounds were documented from day 0 to day 5 using a light microscope with a 4 × objective lens. Finally, the wounded diameter per day was measured to evaluate cell migration ability.

## 2.3 Statistical analysis

The cell viability at gradient concentrations of curcumin was shown by a heatmap visualization technique with support of TBtools software [146]. The correlation analysis of IκB and $IC_{50}$ or IκB and cell viability was evaluated by *Pearson* or *Spearman's* rank correlation (according to the data's

normality or not) and presented as correlation coefficient ($r_s$). The difference of $IC_{50}$ between two different mutation status or MSI status was compared using *student t-test*, and presented as mean ± standard deviation (SD). However, the difference of $IC_{50}$, cell death, IκB level after curcumin treatment, as well as cell cycle within ≥ 3 groups was compared using ANOVA analysis, similarly presented as mean ± SD. In addition, the migration ability between non-drug and curcumin treatment was analyzed by linear regression, and shown as slopes of regression line. The predictive ability for curcumin sensitivity was performed using Receiver Operating Characteristic (ROC) curve, and presented by the area under the ROC curve (AUC).

*P*-values lower than 0.05 were considered significant. All experiments were independently repeated for at least three measurements. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, United States).

# 3. Results

## 3.1 Patients and tumor characteristics

In general, 32 low-passage cell lines established from 30 CC patients and 2 RC patients were used in this study. CRC patients had a mean age of 66 years with a range from 26 to 98 years, and consisted of 15 males and 17 females. In detail, the patient and tumor data also include classification, molecular type as well as somatic mutations. The percentage of Union Internationale Contre le Cancer (UICC) stage was 40.63% for stage I - II and 56.25% for stage III - IV. After molecular diagnosis, around 60% of the study population were MSS and 40% MSI. Moreover, except 2 untested tissues, 56.25% of all specimens were TP53 mutated; around 60% had an APC mutation; 43.75% were K-Ras, 28.13% B-Raf, 15.62% PIK3CA as well as 12.50% ATM mutated (Table 2).

Table 2. Patient characteristics

| **Variables** | | **n** | **%** |
|---|---|---|---|
| Age (years) * | | 32 | 66.34 ± 17.85 |
| Gender | male | 15 | 46.88 |
| | female | 17 | 53.13 |
| Sample location | colon | 30 | 93.75 |
| | rectum | 2 | 6.25 |
| T stage | T0-T2 | 4 | 12.50 |
| | T3-T4 | 28 | 87.50 |
| N stage | N0 | 14 | 43.75 |
| | N1-2 | 18 | 56.25 |
| M stage | M0 | 22 | 68.75 |
| | M1 | 9 | 28.13 |
| | Mx | 1 | 3.13 |
| R stage | R0 | 23 | 71.88 |
| | R1-2 | 8 | 25.00 |

| | | | |
|---|---|---|---|
| | unclear | 1 | 3.13 |
| L stage | L0 | 21 | 65.63 |
| | L1 | 9 | 28.13 |
| | unclear | 2 | 6.25 |
| V stage | V0 | 19 | 59.38 |
| | V1-2 | 11 | 34.38 |
| | unclear | 2 | 6.25 |
| UICC stage | I-II | 13 | 40.63 |
| | III-IV | 18 | 56.25 |
| | unclear | 1 | 3.13 |
| MSI status | MSS | 19 | 59.38 |
| | MSI | 13 | 40.63 |
| TP53 | wt | 12 | 37.50 |
| | mut | 18 | 56.25 |
| | unclear | 2 | 6.25 |
| APC | wt | 11 | 34.38 |
| | mut | 19 | 59.38 |
| | unclear | 2 | 6.25 |
| K-Ras | wt | 16 | 50.00 |
| | mut | 14 | 43.75 |
| | unclear | 2 | 6.25 |
| B-Raf | wt | 21 | 65.63 |
| | mut | 9 | 28.13 |
| | unclear | 2 | 6.25 |
| PIK3CA | wt | 25 | 78.13 |
| | mut | 5 | 15.62 |
| | unclear | 2 | 6.25 |
| ATM | wt | 26 | 81.25 |
| | mut | 4 | 12.50 |
| | unclear | 2 | 6.25 |

* age was presented as mean ± SD; wt, wild type; mut, mutant type.

## 3.2 Curcumin sensitivity in CRC cells

All CRC cell lines were treated with six concentrations of curcumin (1μM, 5μM, 10μM, 15μM, 20μM and 25μM). A minority of CRC cell lines responded at a concentration of 5μM curcumin. In addition, nearly half of the tested CRC cell lines displayed around 50% cell viability after 10μM drug treatment. However, up to 20% CRC cell lines are still resistant to the highest drug concentration used (Figure 10).

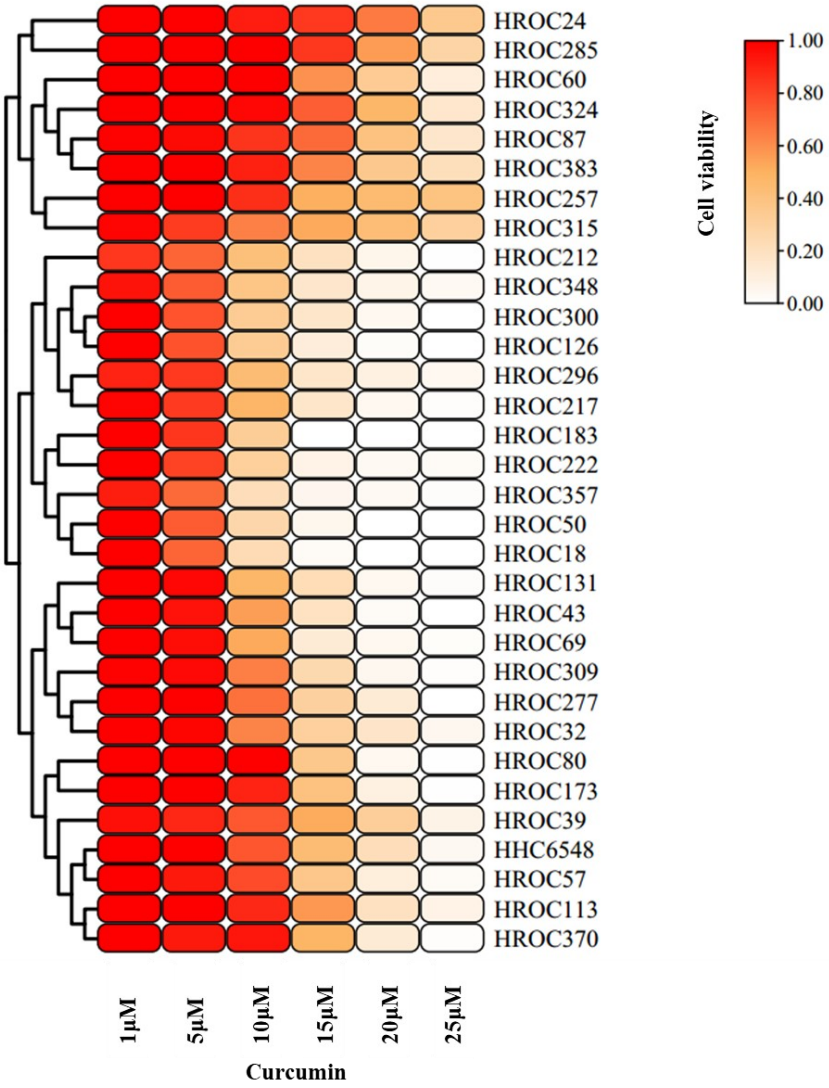



Figure 10. The 32 CRC cell lines were tested for curcumin sensitivity by a long-term proliferation assay. Each cell line test was repeated at least three times with technical triplicates. The heatmap was plotted with the help of TBtools software.

## 3.3 $IC_{50}$ of curcumin and IκB level in CRC cells

The $IC_{50}$ of curcumin showed a range from 6.69μM to 18.49μM. This result identified the most sensitive five cell lines including the lowest $IC_{50}$ of curcumin: HROC18 ($IC_{50}$ = 6.69μM), closely followed by 6.97μM for HROC357, 7.02μM for HROC50, 7.60μM for HROC222 as well as 7.72μM for HROC126. On the contrary, the several top resistant cell lines were HROC24 with an $IC_{50}$ of 18.49μM, 18.27μM for HROC285, 17.52μM for HROC324 and 16.39μM for HROC87 (Figure 11).

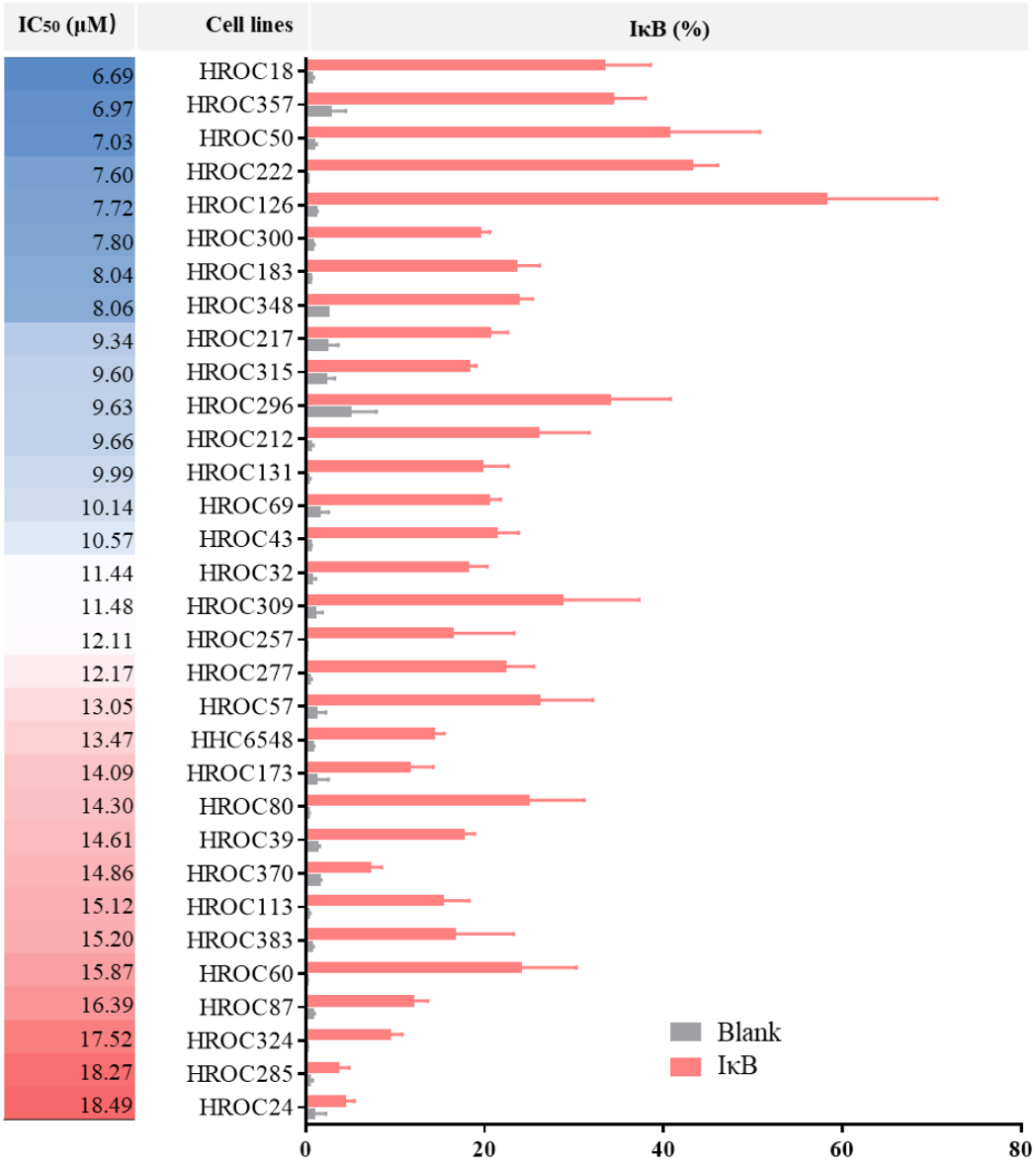


Figure 11. The $IC_{50}$ of curcumin and the IκB expression level in CRC cell lines of the HROC collection. The $IC_{50}$ of curcumin was calculated with the support of GraphPad Prism 6 software. The IκB level in CRC cells was determined using flow cytometry. Blank group (grey histograms) represented staining with an irrelevant control antibody and the IκB group (red histograms) represented staining of IκB antibody; the bars were expressed as standard deviation (SD) of the mean.

Next, the inhibitors of the IκB level in all 32 CRC cell lines were directly measured by flow cytometry. The IκB expression level in CRC cell lines differed from the highest value 56.96% to the lowest value of 3.2%. Interestingly, it has been found that the above mentioned most sensitive five cell lines had very high IκB levels with 56.96% in HROC126, 42.82% in HROC222, 39.72% in HROC50, 32.73% in HROC18 as well as 31.54% in the HROC357 cell line. On the other hand, the curcumin resistant cell lines expressed very low IκB levels, only 3.20% in HROC285, 3.43% in HROC24, 9.23% in HROC324 and 11.28% in the HROC87 cell line. Notably, a marked difference in IκB levels between sensitive and resistant CRC cell lines was observed in this analysis (Figure 11).

## 3.4 Correlation analysis between $IC_{50}$ of curcumin and IκB in HROC cell lines

In order to identify the correlation between IκB and $IC_{50}$ of curcumin in CRC cell lines, a *Pearson* correlation analysis was performed. The correlation analysis showed that $IC_{50}$ of curcumin in CRC cell lines was significantly negatively correlated with the level of IκB in cells, and with a coefficient of -0.72, $P < 0.001$ (Figure 12). This strongly proposed that CRC cell lines with high IκB levels are likely more sensitive to curcumin, conversely cell lines with lower IκB levels are more resistant to curcumin.

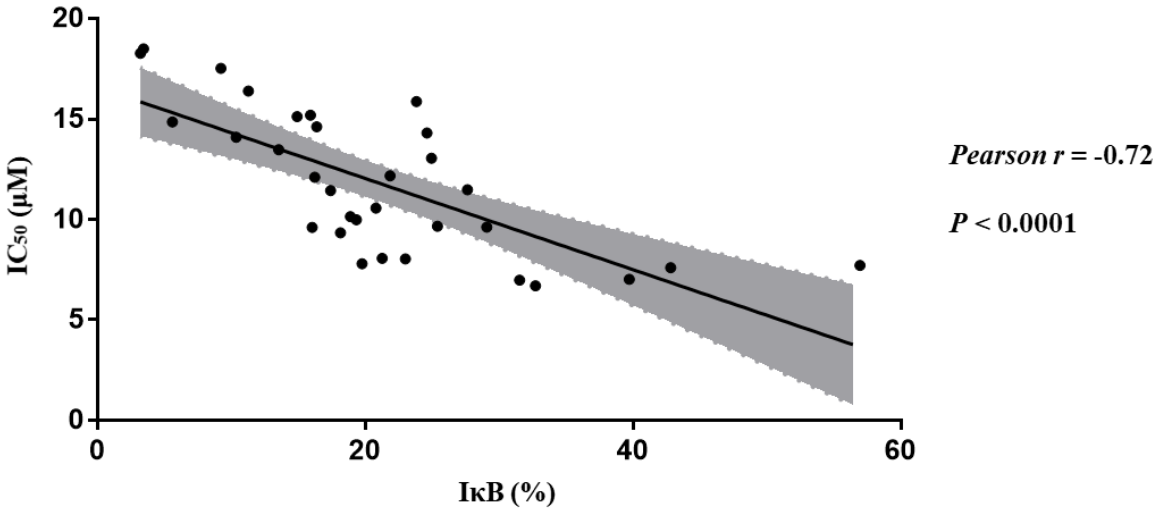
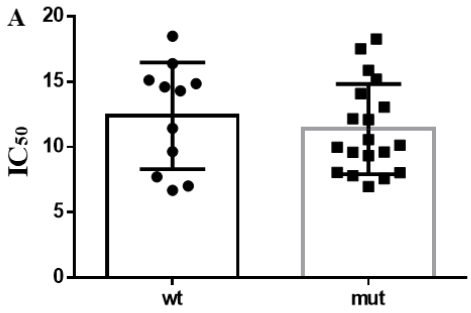


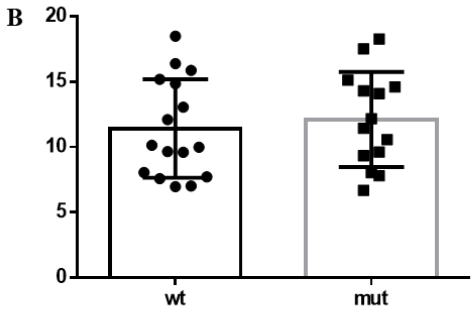

Figure 12. The $IC_{50}$ of curcumin correlates with IκB expression levels of CRC cell lines. This correlation analysis was performed by *Pearson* analysis, and the correlation coefficient ($r_s$) was identified as the measurement of the strength of the relationship between $IC_{50}$ of curcumin and IκB expression level in CRC cell lines.

## 3.5 Genomic profiles and curcumin sensitivity in CRC cell lines

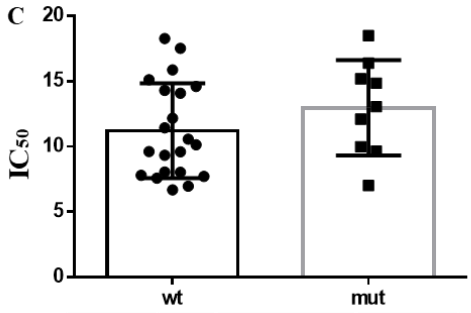
Mutations in APC, K-Ras, B-Raf, PIK3CA, TP53 as well as ATM genes are commonly evaluated as biomarkers in CRC. In present study, the $IC_{50}$ of curcumin was also analyzed based on mutant or wild type of APC, K-Ras, B-Raf, PIK3CA, TP53 as well as ATM, respectively, in CRC cell lines. Overall, these results showed that higher $IC_{50}$ of curcumin were found in CRC cell lines with wild type in the genes APC, and with mutant status of the genes B-Raf, K-Ras, PIK3CA, TP53 as well as ATM (Figure 13). However, these mutational signatures did not significantly discriminate curcumin-sensitive from -resistant CRC cell lines.



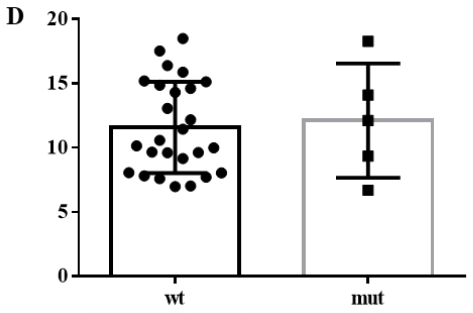

| APC | n | Mean ± SD | *P*-value |
|---|---|---|---|
| wt | 11 | 12.39 ± 1.23 | 0.470 |
| mut | 19 | 11.37 ± 0.79 | |



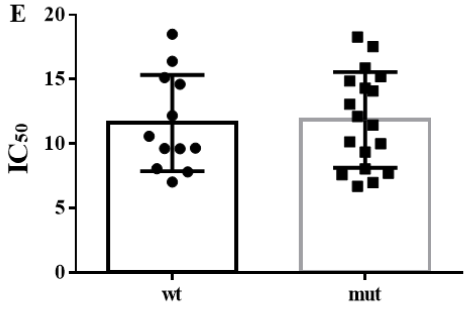

| K-Ras | n | Mean ± SD | *P*-value |
|---|---|---|---|
| wt | 16 | 11.42 ± 0.94 | 0.614 |
| mut | 14 | 12.11 ± 0.97 | |



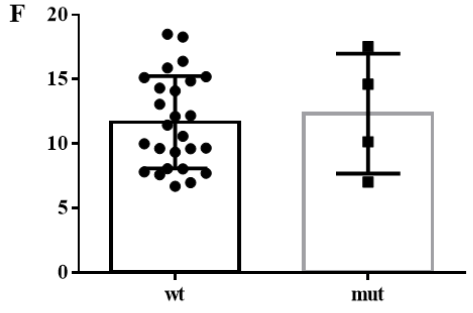

| B-Raf | n | Mean ± SD | *P*-value |
|---|---|---|---|
| wt | 21 | 11.22 ± 0.79 | 0.234 |
| mut | 9 | 12.98 ± 1.22 | |




| PIK3CA | n | Mean ± SD | *P*-value |
|---|---|---|---|
| wt | 25 | 11.67 ± 0.72 | 0.816 |
| mut | 5 | 12.10 ± 1.99 | |




| TP53 | n | Mean ± SD | *P*-value |
|---|---|---|---|
| wt | 12 | 11.59 ± 1.08 | 0.858 |
| mut | 18 | 11.84 ± 0.88 | |

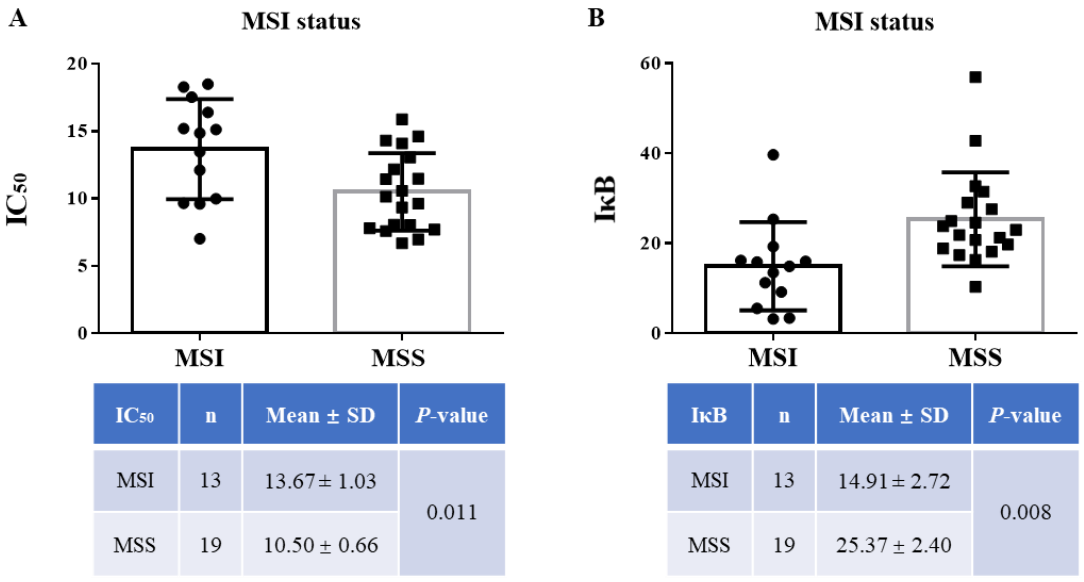



| ATM | n | Mean ± SD | *P*-value |
|---|---|---|---|
| wt | 26 | 11.65 ± 0.70 | 0.740 |
| mut | 4 | 12.32 ± 2.33 | |

Figure 13. The $IC_{50}$ (μM) of CRC cell lines to curcumin in cell lines with different mutational profiles for APC (A), K-Ras (B), B-Raf (C), PIK3CA (D), TP53 (E) and ATM (F). The t-test was performed to compare the mean value between wild type and mutant genes. Wt, wild type; mut, mutant type.

## 3.6 MSI status and curcumin sensitivity in CRC cell lines

Stratified by MSI status, the $IC_{50}$ of curcumin in MSS CRC cell lines is significantly lower than in MSI CRC (MSS vs. MSI, 10.50 ± 0.66μM vs. 13.67 ± 1.03μM, $P$ = 0.011) (Figure 14A). This signified that CRC cell lines with MSS are more sensitive to curcumin, while CRC cell lines with MSI are less responding to curcumin. In addition, the IκB level in CRC cell lines was also compared by MSI status. Interestingly, the analysis showed that the mean IκB level in MSS CRC cell lines is significantly higher compared to MSI CRC cell lines (MSS vs. MSI, 25.37 ± 2.40μM vs. 14.91 ± 2.72μM, $P$ = 0.008) (Figure 14B).



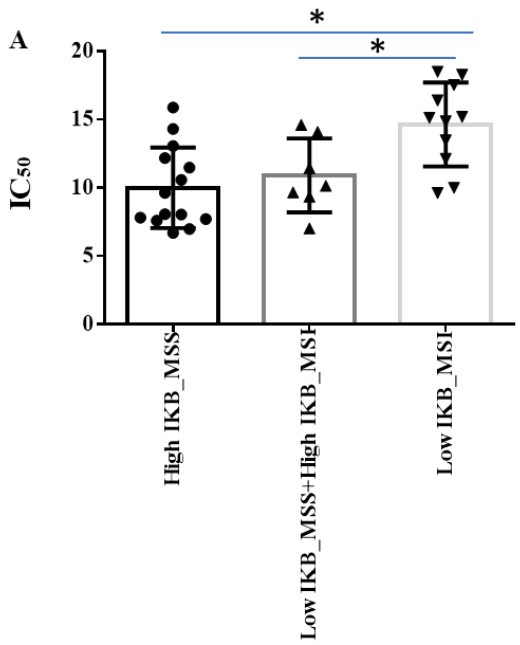

| $IC_{50}$ | n | Mean ± SD | *P*-value |
|---|---|---|---|
| MSI | 13 | 13.67 ± 1.03 | 0.011 |
| MSS | 19 | 10.50 ± 0.66 | |

| IκB | n | Mean ± SD | *P*-value |
|---|---|---|---|
| MSI | 13 | 14.91 ± 2.72 | 0.008 |
| MSS | 19 | 25.37 ± 2.40 | |

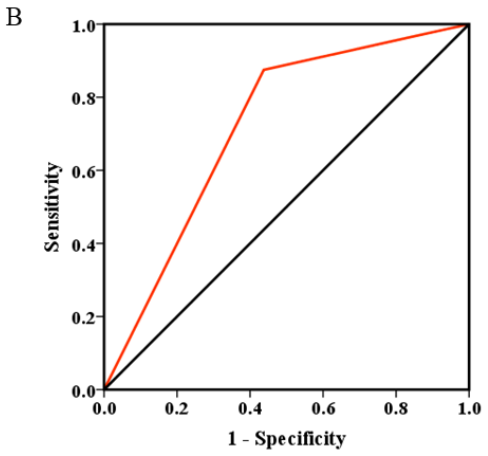
Figure 14. The comparison of $IC_{50}$ (μM) of curcumin between MSI and MSS CRC cell lines (A). The comparison of IκB level in MSI and MSS CRC cell lines (B).

## 3.7 Prediction of a subset of CRC cell lines with sensitivity to curcumin

According to MSI status and IκB level in cells, all CRC cell lines were further divided into three groups: MSS cell lines with high IκB level (High IκB_MSS), MSS cell lines with low IκB level or MSI cell lines with high IκB level (Low IκB_MSS or High IκB_MSI) as well as MSI cell lines with low IκB level (Low IκB_MSI). After pairwise comparisons, no difference between High IκB_MSS and Low IκB_MSS or High IκB_MSI was observed for $IC_{50}$ of curcumin; but these two groups showed significantly lower $IC_{50}$ compared with the Low IκB_MSI group, respectively. Therefore, both groups of High IκB_MSS and Low IκB_MSS or High IκB_MSI was combined as an entire one (High IκB or MSS) (Figure 15A).




| $IC_{50}$ | n | Mean ± SD |
|---|---|---|
| High IκB_MSS | 14 | 10.00 ± 2.95 |
| Low IκB_MSS / High IκB_MSI | 7 | 10.90 ± 2.70 |
| Low IκB_MSI | 11 | 14.64±3.08 |




| AUC | 95%CI | *P*-value |
|---|---|---|
| 0.72 | 0.54-0.90 | 0.035 |

Figure 15. The IκB level in CRC cells and their MSI status were used to predict the CRC cell lines sensitivity to curcumin. The $IC_{50}$ of curcumin were compared among CRC cell lines with High IκB_MSS, Low IκB_MSS or High IκB_MSI as well as Low IκB_MSI using multiple comparison of ANOVA (A). The predictive ability of IκB level in CRC cells and MSI status was evaluated by receiver operating characteristic (ROC) and presented by the area under the ROC curve (AUC) (B).

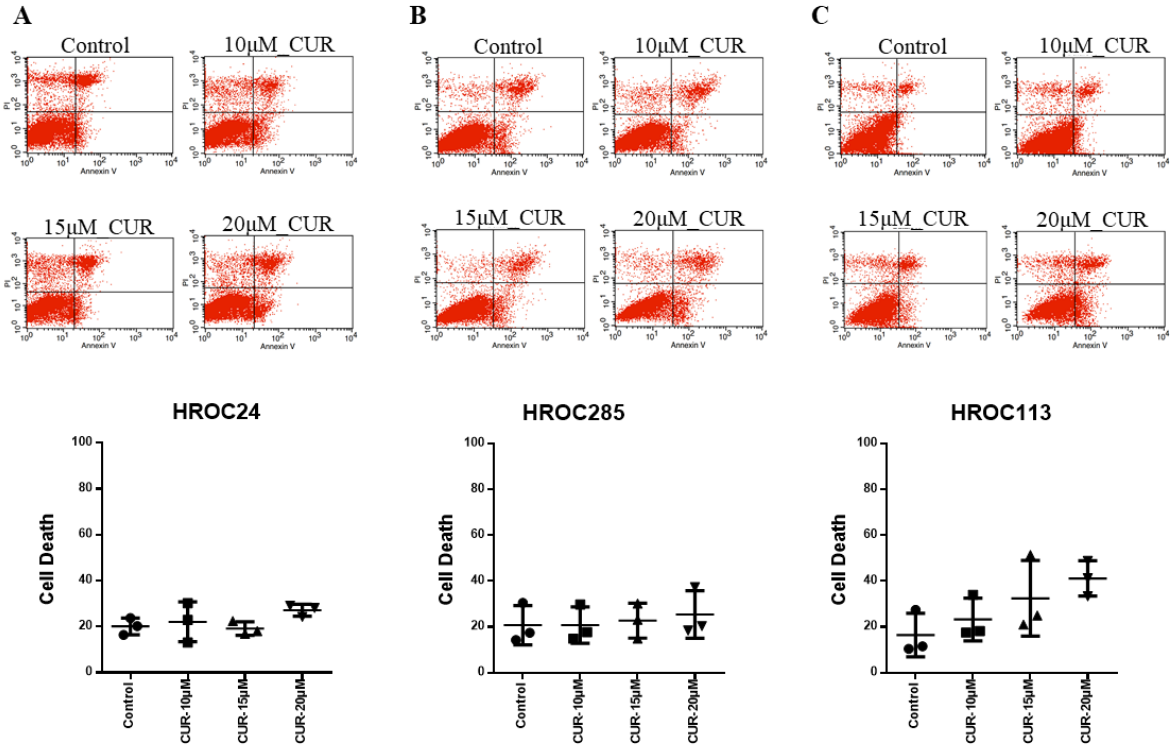
In order to predict the curcumin sensitivity, all CRC cell lines were divided into a curcumin-resistance group and a curcumin-sensitivity group based on the median $IC_{50}$ of curcumin (Figure 11). Then, IκB level and MSI status were used as the biomarkers to predict the sensitivity of CRC cell lines to curcumin.

Finally, the AUC of ROC presented a significantly predictive value (AUC = 0.72, 95%CI: 0.54 - 0.90, $P$ = 0.035) with a 72% probability which could correctly distinguish curcumin sensitive CRC cell lines form those resistant ones (Figure 14B).

## 3.8 Cell death after curcumin treatment

In order to further examine the different sensitivity between High IκB or MSS group and Low IκB_MSI group, three cell lines from these two groups were respectively selected. HROC24, HROC113 and HROC285 cell lines with the features of Low IκB and MSI are regarded as the curcumin-resistance CRC cell lines. On the contrary, the HROC18, HROC357 and HROC69 cell lines with the characteristics of High IκB and MSS are considered as the curcumin-sensitivity CRC cell line.

These cell lines were pre-treated using non-drug (control), 10μM curcumin, 15μM curcumin and 20μM curcumin for 72 hours, then the difference between control and curcumin treatment was analyzed. As expected, cell death in HROC24, HROC113 and HROC285 was not significantly different in comparison to the controls, even at a concentration of 20μM curcumin. However, in the curcumin-sensitive group, cell death was significantly higher in all three cell lines compared to the control group ($P < 0.05$), even with an obvious positive dose response. The strong curcumin-response in HROC357 started at 10μM curcumin ($P < 0.05$), and the cell deaths in HROC357 and HROC69 cell lines went up to 80% when treated by 20μM curcumin ($P < 0.05$) (Figure 16).



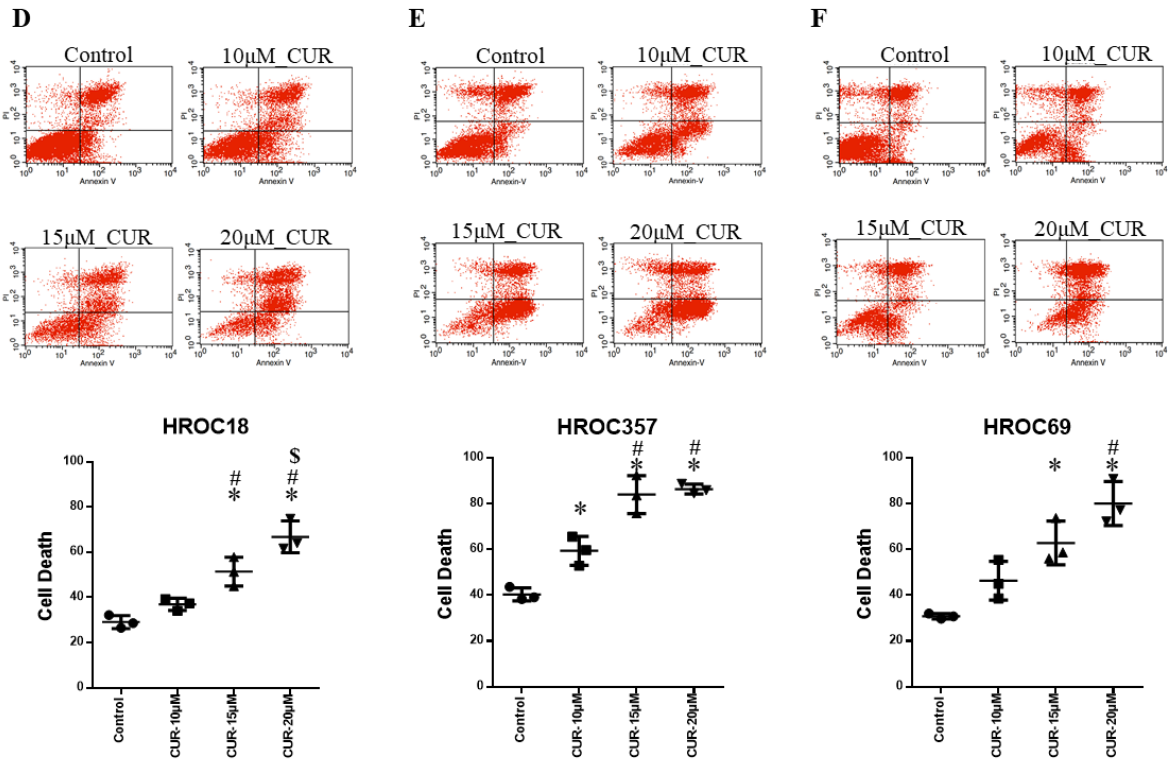



Figure 16. The cell death was measured in three curcumin-resistance CRC cell lines (HROC18, HROC69 and HROC357) and three curcumin-sensitivity CRC cell lines (HROC24, HROC285 and HROC113) after treating with 10μM, 15μM, 20μM curcumin. The cell death of these cells was detected by flow cytometry. *, compared to control; #, compared to 10μM curcumin treatment; $, compared to 15μM curcumin treatment; all these markers showed $P < 0.05$. CUR, curcumin.

## 3.9 Migration after curcumin treatment

Two curcumin sensitive cell lines (HROC18 and HROC357) and two curcumin resistant cell lines (HROC24 and HROC87) were pre-treated by non-drug (control) and 10μM curcumin for incubation overnight, after that the wounded diameters were measured daily for up to 5 days. Similarly, compared to the controls, the migration in HROC24, HROC87 failed to show statistical significance. However, in the curcumin-sensitive group, the migration ability of cells after 10μM curcumin pre-treatment was significantly lower compared to control, $P < 0.001$ (Figure 17).

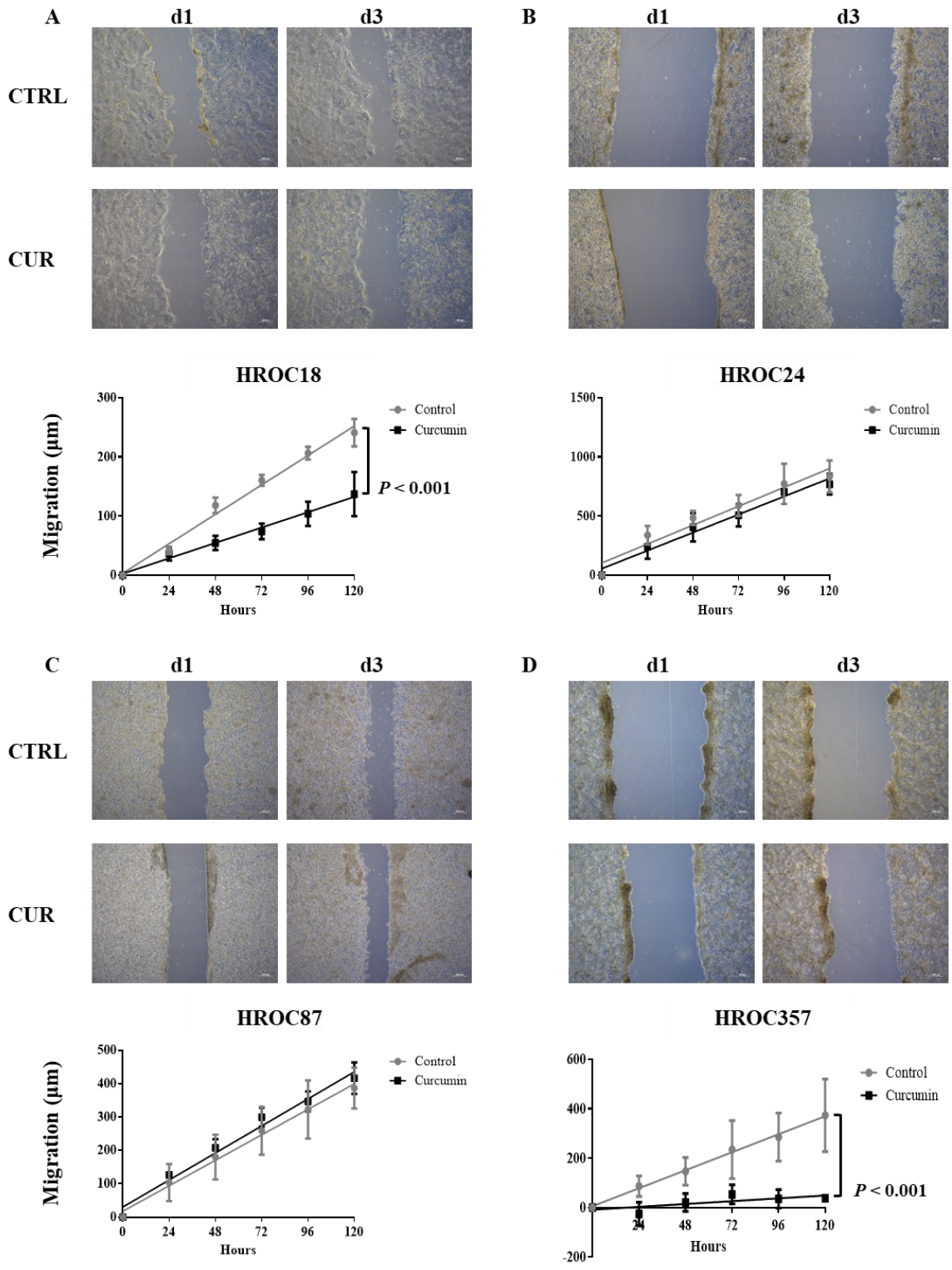



Figure 17. Migration test in two curcumin-resistant CRC cell lines (HROC24 and HROC87) and two curcumin-sensitive CRC cell lines (HROC18 and HROC357) treated by 10μM curcumin, for 24 hours. Migration was detected by wound healing assay. The statistical difference analysis was performed by linear regression, $P < 0.001$, compared to control (non-drug treatment). CUR, curcumin.

## 3.10 Cell cycle after curcumin treatment

This present study also evaluated the cell cycle using two curcumin sensitive and two resistant CRC cell lines. After 72 hours treatment with 20μM curcumin, the data showed that curcumin made around 20% HROC24 and HROC285 cells arrest at the S stage ($P < 0.05$, Figure 18A and B), but more than 50% HROC69 and HROC357 cells arrested at the G2/M stage ($P < 0.05$, Figure 18C and D).

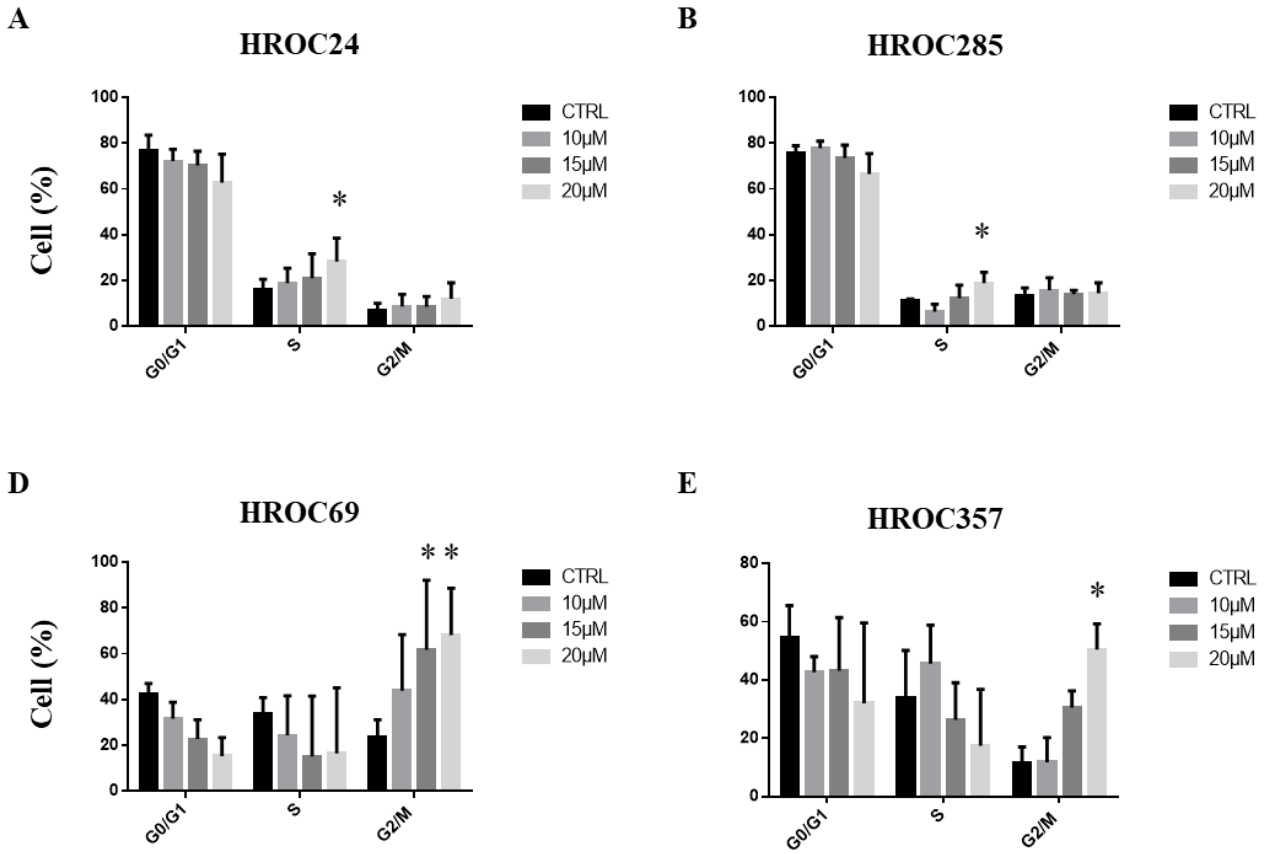
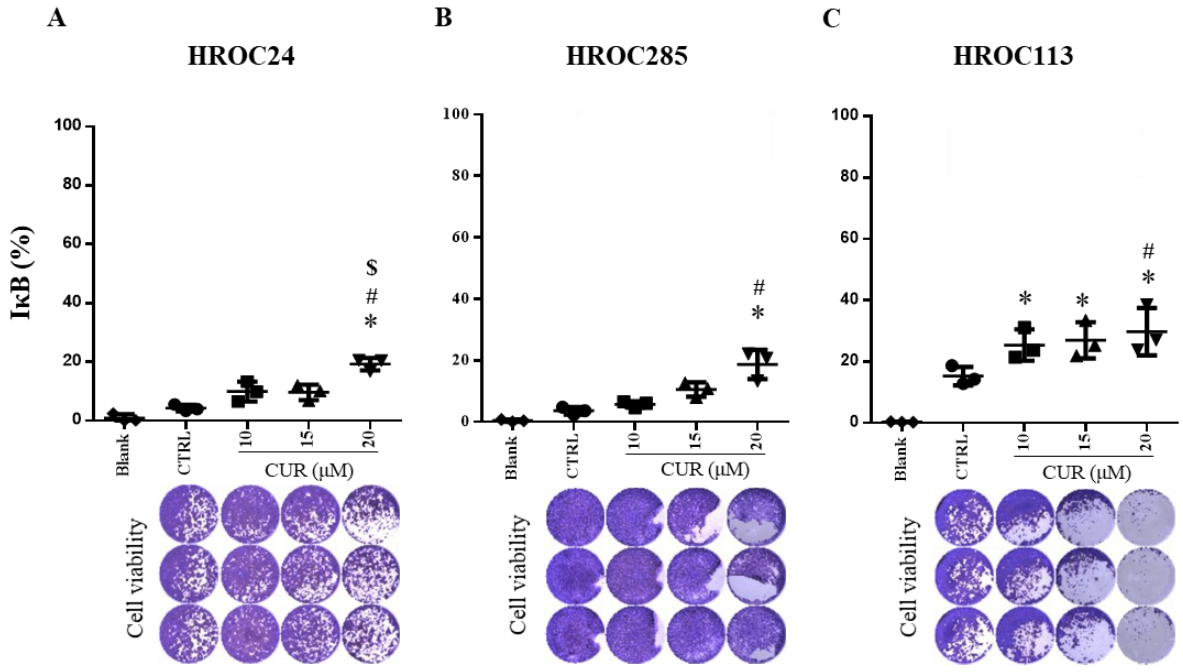



Figure 18. Cell cycle in two curcumin-resistant CRC cell lines and two curcumin-sensitive CRC cell lines treated by 10, 15, 20μM curcumin. Cell cycle was detected by flow cytometry. *, compared to control (non-curcumin treatment, CTRL), $P < 0.05$.

## 3.11 Increased IκB after curcumin treatment

Curcumin-sensitive and -resistant cell lines were also incubated using non-drug (control), 10μM curcumin, 15μM curcumin and 20μM curcumin for 24 hours, then the IκB level in cells was detected. In general, a growing trend of IκB was observed in all these 6 CRC cell lines with increasing curcumin concentrations. The IκB level in curcumin-resistant cell lines is significantly lower in the non-drug treatment group (compare Figure 19 A, B and C to D, E and F). After treatment with 20μM curcumin only around 20% increase in IκB ($P < 0.05$, Figure 19A-C) could be detected. However, for the sensitive cell lines, a significant difference between control and curcumin treatments starts at 10μM curcumin ($P < 0.05$), with even up to 70% increase of IκB triggered by 20μM curcumin ($P < 0.05$, Figure 19D-E).



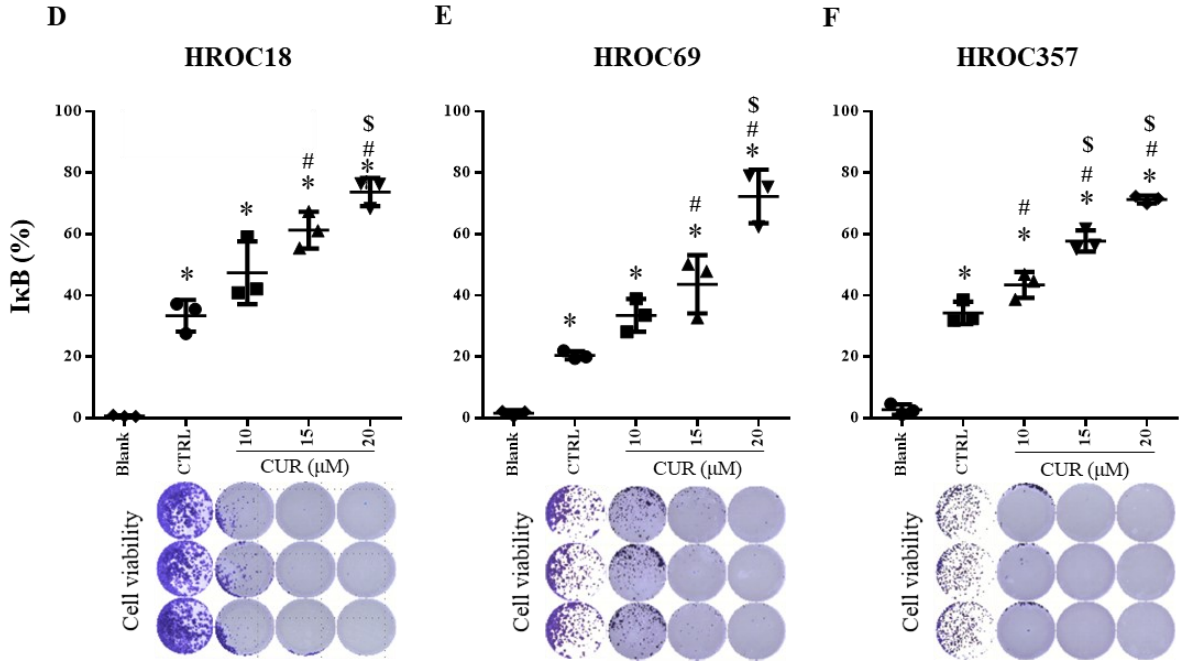



Figure 19. The IκB level in three curcumin-resistant and three curcumin-sensitive CRC cell lines treated by 10μM, 15μM, 20μM curcumin. IκB level of these cells were detected by flow cytometry. *, compared to Blank; #, compared to control (CTRL); $, compared to 10μM curcumin treatment; all these markers mean $P < 0.05$. CUR, curcumin.

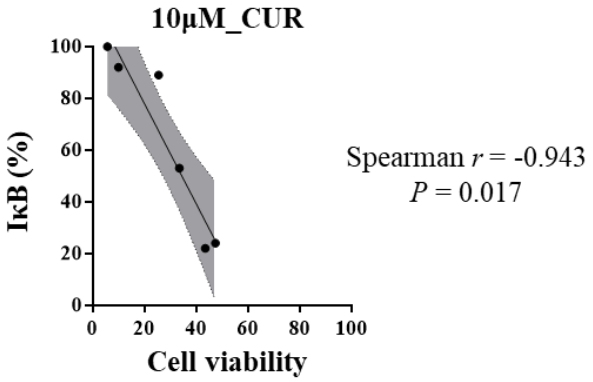
## 3.12 Correlation analysis between cell viability and IκB after curcumin treatment

After curcumin treatment (10μM, 15μM and 20μM, respectively), the correlation analyses between cell viability and increased IκB levels were done in 6 CRC cell lines (three curcumin resistant cell lines: HROC24, HROC113 and HROC285; three curcumin sensitive cell lines: HROC18, HROC69 and HROC357). At first, with 10μM curcumin treatment, a strongly significant correlation between cell viability and IκB level in CRC cell lines was observed (Figure 20A), with a very high value of coefficient ($r_s$ = -0.943, $P$ = 0.017). This means that the cell viability is negatively associated with the IκB level in CRC cell lines after 10μM curcumin treatment. Similarly, this kind of negative

correlation continued to show with 15μM (Figure 20B; $r_s$ = -0.986 ($P$ < 0.0001)) and 20μM curcumin (Figure 20C; $r_s$ = -0.928 ($P$ = 0.006)) groups.

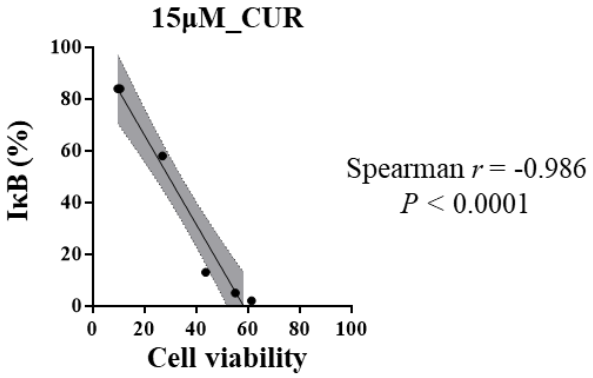
**A**

| Cell lines | CUR 10μM | |
|---|---|---|
| | IκB (%) | Cell viability (%) |
| HROC357 | 43.46 | 21.78 |
| HROC18 | 47.37 | 23.59 |
| HROC69 | 33.49 | 52.66 |
| HROC113 | 25.42 | 88.73 |
| HROC24 | 9.92 | 91.79 |
| HROC285 | 5.74 | 100.00 |




**B**

| Cell lines | CUR 15μM | |
|---|---|---|
| | IκB (%) | Cell viability (%) |
| HROC18 | 61.30 | 2.41 |
| HROC357 | 55.01 | 5.40 |
| HROC69 | 43.60 | 13.00 |
| HROC113 | 26.95 | 57.70 |
| HROC24 | 9.69 | 84.12 |
| HROC285 | 10.55 | 84.31 |




**C**

| Cell lines | CUR 20μM | |
|---|---|---|
| | IκB (%) | Cell viability (%) |
| HROC18 | 73.70 | 0.00 |
| HROC357 | 67.97 | 3.69 |
| HROC69 | 72.28 | 4.40 |
| HROC113 | 29.79 | 19.57 |
| HROC285 | 18.68 | 56.19 |
| HROC24 | 19.26 | 66.04 |

Low High

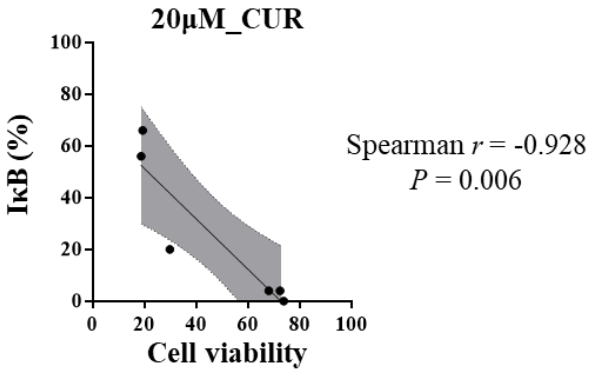



Figure 20. Correlation analysis of cell viability and IκB after treatment with 10μM (A), 15μM (B) and 20μM (C) curcumin. These correlation analyses were performed by *Spearman* correlation analysis. CUR, curcumin.

# 4. Discussion

The present study is the first report screening a subset of CRC cell lines with sensitivity to curcumin and identified a possible biomarker which is associated with the curcumin sensitivity in CRC cell lines. In detail, 32 CRC cell lines were stratified for MSI status, APC, K-Ras, B-Raf, PIK3CA, TP53 and ATM mutations, and tested sensitivity to curcumin. In the preliminary sensitivity test, it was found that CRC cell lines of this panel have different sensitivities to curcumin.

In the next exploration for a biomarker of curcumin sensitivity, and taking into account that mutations in APC, K-Ras, B-Raf, PIK3CA, TP53 and ATM are usually considered as relevant CRC biomarkers [147-153], the difference between wild type and mutations of these genes was compared in the different cell lines, but mutations in these genes were not associated with curcumin sensitivity or resistance.

Moreover, MSI status or MMR genes was also regarded as an important biomarker in cancer progression, prognostic outcome, even adjustment of adjuvant chemotherapy [154-157]. In this case, analyzing the curcumin sensitivity stratified by MSI status seems necessary. This result showed that most MSI CRC cell lines are more resistant to curcumin, whereas most MSS CRC cell lines are sensitive to curcumin.

On the other hand, IκB was considered as a key inhibitory protein of NF-κB in the NF-κB signaling pathway, and, for the first time, was identified as an important biomarker to predict the sensitivity to curcumin treatment. The present study showed that curcumin-sensitive cell lines express

significantly higher levels of IκB even without treatment, while curcumin-resistant cell lines frequently express low IκB levels.

Interestingly, when combining IκB levels with the MSI status as biomarker for predicting curcumin sensitivity, it could correctly distinguish the curcumin sensitive CRC cell lines from curcumin resistant CRC cell lines with approximately 72% predictive power.

## 4.1 Drawbacks of current chemo-drugs

Nowadays, Fluorouracil is still the backbone for the treatment of mCRC. And these combinations of chemo-drugs have achieved clear improvement of mCRC patients' outcome. Establishing a chemotherapy regimen of fluorouracil combined with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) or even these three chemo-drug combinations (FOLFOXIRI) is a treatment regimen that can produce better anti-tumor effect [158-162].

However, these treatment modalities are usually accompanied with major side effects. As Katona et al. [163] reported, 5-FU as the standard treatment care for CRC, however, its efficacy is limited due to toxic side effects, such as mucositis, diarrhea, myelotoxicity, angina pectoris, hypertension.

In addition, the oxaliplatin chemotherapy was also regarded as classical first line chemo-drug for CRC treatment. However, the main drawback of the addition of oxaliplatin chemotherapy is the development of cumulative sensory neuropathy [164-166]. Besides, more cases have been reported that patients with CRC are hypersensitive to oxaliplatin, and even if it rarely occurs, it is considered idiopathic [167, 168].

One more first-line chemo-drug for CRC treatments, irinotecan is also accompanied by severe toxicities, such as neutropenia and diarrhea which can lead to interruption or cessation, thereby, jeopardizing prognosis and life quality of CRC patients [169]. Cardiotoxicity is frequently seen in CRC patients treated with chemo-drugs, such as 5-FU, capecitabine [170, 171], capecitabine in conjunction with oxaliplatin [172] as well as FOLFOX [173, 174].

Although, a large number of clinical trials have benefited from combination therapy, in terms of the overall quality of life of patients, multi-drug combinations are still unsatisfactory due to aggravated side effects. As Montagnani et al. reported, FOLFOXIRI confers significant benefit in progression-free survival, overall survival, response and R0 resection rates but is more toxic compared with FOLFIRI [175].

Moreover, chemo-drug resistance was still an additional major challenge in the clinical treatment for advanced CRC patients as a remarkable progress in tumors [176-180]. However, the mechanism for chemotherapy resistance is not fully known. Gong et al. [181] reported that Nuclear factor-carotenoid 2-related factor 2 (Nrf2), a main regulator of cells against oxidative and electrophilic stress, is considered to be the possible factor for 5-FU resistance. In addition, a recent study reported that 5-FU resistance is associated with the transcriptional coactivator YAP [182]. In a linear model for predicting chemotherapy resistance and survival rate, the eight-lncRNA signature is defined as a new biomarker for predicting FOLFOX chemotherapy response and prognosis of patients with mCRC [183]. In the exploration of oxaliplatin resistance, Lan et al. [184] found that tumor-associated macrophages possibly promote resistance of drug via METTL3-mediated $m^6$ A of TRAF5 in CRC.

The KRAS, NRAS, and BRAF mutation status of patients with mCRC is the most important determinant of cetuximab resistance. The data analysis showed that all mCRC patients with BRAF mutations or multiple mutation patterns were cetuximab non-responders. A mutation in one of KRAS, NRAS, and BRAF was significantly associated with poorer response rate (7.1%) as well as progression-free survival (8.0 months) when compared to all Ras wild-type mCRC patients (74.4% and 11.6 months) [185]. Roock et al. [186] further confirmed that BRAF, NRAS, and additional PIK3CA exon 20 mutations are significantly associated with a low response rate to cetuximab.

Not only mentioned above, a majority of researchers are dedicating to overcoming or reducing drug resistance in the chemotherapy of CRC. In short, they try to predict the biomarkers leading to chemo-drug resistance or they screen a specific subset of CRC with high sensitivity or resistance to a certain chemo-drug or multiple chemo-drug combinations.

However, to date, these issues in chemotherapy including toxic side effects and drug resistance, still are known as a very challenging problem limiting the efficacy of therapies and impacting the CRC patient's quality of life [187]. Therefore, more effective treatments are mandatory. Among others, this has triggered for the scientific exploration of natural compounds with anti-cancer properties which possess fewer side effects.

## 4.2 Safety and effectiveness of curcumin

As mentioned before, curcumin, one such diet-derived agent, is expected to become a promising candidate for drug use. In terms of safety, few toxicities have been reported in previous clinical studies. Pharmacokinetics and safety of curcumin have been widely described in healthy volunteer

and clinical populations [188]. A study of curcumin dose escalation (standard powder extract and uniformly ground, C3 Complextrade trademark, Sabinsa Corporation) was conducted to evaluate the maximum tolerated dose and safety of a single dose [189]. In this study, 24 healthy volunteers were included and administered increasing doses from 500 to 12,000mg. 7/24 experienced only a very mild toxicity which was not associated with the increased dose of curcumin. The tolerance and safety of curcumin in high single oral dose application appears to be excellent, even at very high doses such as 12g/day in humans [189]. Moreover, window of opportunity studies for CRC patients have determined both safety and tolerance for patients awaiting surgical resection of cancer [132, 190]. After oral administration, pharmacologically active concentrations of curcumin are detectable in blood and bowel mucosa [132]. This phase I clinical trial of oral curcumin included 15 patients with advanced CRC refractory to standard chemotherapies. These patients consumed curcumin doses between 0.45 and 3.6g daily for up to 4 months, and the biological activity was detectable. The study suggested that curcuma extract can be administered safely to patients with advanced CRC at doses of up to 2.2g daily [132].

The potential of curcumin as a low toxicity adjuvant for chemotherapy has also been studied in preclinical models for CRC treatment [188]. The powerful activity of curcumin as well as novel curcumin in cancer has been completed in phase I clinical trials involving efficacy and systemic circulation, and initiated the ongoing phase II and phase III clinical trials in multiple centers of research around the world [132, 191-194].

In terms of effectiveness, the present study determined curcumin's efficacy in decreasing cell proliferation, inducing cell death as well as mediating cell cycle arrest in G2/M stage *in vitro*, which

is most possibly linked to the NF-κB pathway. Furthermore, a lot of supportive studies also have illustrated the effectiveness of curcumin *in vivo* [135, 195-197] and in patient-derived *ex vivo* models [198, 199] .

It is worth noting that chemo-drugs combined with curcumin have an additive or synergistic effect on CRC patients and are a well-tolerated chemotherapy adjunct. James et al. [198] reported, that curcumin alone and curcumin in combination with oxaliplatin / 5-FU significantly decreased the spheroid number in cancer stem cell (CSC) models of patient-derived CRC liver metastases and the number of cells with high aldehyde dehydrogenase activity. Furthermore, adding curcumin to oxaliplatin / 5-FU can increase the anti-proliferation and pro-apoptosis effects for these CRC patient-derived explants. In phase I clinical trials, a dose escalation study revealed curcumin is a safe and tolerable adjunct to FOLFOX chemotherapy in patients with CRC liver metastases [198]. Several similar studies also showed that curcumin significantly induced apoptosis and decreased proliferation when combined with oxaliplatin in CRC cell lines [200] and *in vivo* using a HCT116 xenograft mouse model [135]. In a phase IIa open-labelled randomized controlled trial, Howells et al. [188] evaluated the safety, efficacy, survival outcome, neurotoxicity and plasma curcuminoids level in patients with a histological diagnosis of mCRC treated by folinic acid + 5-fluorouracil + oxaliplatin (FOLFOX) or by FOLFOX + oral curcumin (CUFOX). 28 patients with mCRC were randomly divided into two groups receiving either FOLFOX or CUFOX chemotherapy. This study [188] firstly determined the safety and tolerance of addition of daily oral curcumin to FOLFOX. More than 1.00pmol/ml curcumin glucuronide could be detected in 15/18 cases receiving CUFOX. Compared with the CRC patients receiving FOLFOX, patients receiving CUFOX showed

significantly better overall survival (FOLFOX vs. CUFOX; 200d vs. 502d, respectively). Meanwhile, no significant difference was observed concerning neurotoxicity as well as other toxic side effects [188].

These clinical trials determined that mCRC have a greater sensitivity to the combination of curcumin with chemotherapy. Combination therapy of curcumin and FOLFOX is indisputable for improving the survival benefits in patients with advanced CRC. Regarding the future, curcumin might become a promising chemotherapy under development for the mCRC treatment and comprehensive profiles are also currently being evaluated in most clinical trials.

## 4.3 Improved absorption efficacy of curcumin

### 4.3.1 Improvement of curcumin bioavailability

Although an increasing number of studies have predicted that curcumin will become an effective anticancer candidate drug, it is poorly absorbed and has low bioavailability, which limits its clinical transformation [201]. To overcome this challenging issue in the clinical application of curcumin, a series of innovative forms of curcumin were developed aiming to improve the absorption and achieve better therapeutic effects.

Among these, Theracurmin® was made using a microparticle and surface-controlled drug delivery system, which exhibited more than 40-fold greater bioavailability compared to that of curcumin powder in rats [202]. Then, 30mg Theracurmin® or curcumin powder was orally administered in healthy human volunteers. The absorption efficacy of Theracurmin® was 27-fold higher compared to

that of curcumin powder [202]. In addition, Kanai et al. demonstrated in a controlled clinical trial study that Theracurmin® can safely increase high plasma curcumin levels (up to 210mg) without saturating the absorption system in healthy volunteers [203]. Hence, Theracurmin® could be considered as an advanced tool for further exploring the utility for CRC in clinical trials. A phase I study by Kanai's group has further demonstrated the safety of Theracurmin® in cancer patients [193]. In addition, several preclinical studies also reported that Theracurmin® efficiently inhibits the cell growth, induces cell death, induces cell cycle arrest in human prostate and bladder [204] as well as in pancreatic cancer [205]. However, until now, the clinical applicability of Theracurmin® for patients with CRC remains unclear.

In addition, nanocurcumin preparations, such as liposomal curcumin (Lipocurc™) and lecithinized curcumin delivery system (Meriva®), have achieved an advanced bioavailability for curcumin [135, 206-208].

In a phase I, single-center, open-label study [209], 32 cancer patients were included and divided into two groups. One group of them (n = 26) was treated by an escalated dose from 100 to 300mg/$m^2$ for over 8h; no dose-limiting toxicity was observed. Another group (n = 6) received 300mg/$m^2$ liposomal curcumin infusion for over 6h. Among these cancer patients, one case developed hemolysis; three cases had hemoglobin decrease, but no signs of hemolysis and two patients revealed significant tumor marker responses and transient clinical benefit. Hence, 300mg/$m^2$ liposomal curcumin infusion for 6h is recommended as a starting dose for those heavily pretreated cancer patients. Interestingly, in a comparative study between cancer patients and healthy individuals

following intravenous infusion of Lipocurc™, the plasma curcumin level in cancer patients rose to a greater extent with increasing infusion rate compared to healthy individuals [206].

Another promising nanocurcumin preparation, Meriva® has shown a remarkably higher absorption of curcumin at doses significantly lower than unformulated curcumin [210]. This point was further supported by a controlled clinical trial [208]. In detail, Belcaro et al. [208] recruited 160 cancer patients undergoing chemo- and radiotherapy following surgery. Then, Meriva® was used to assess the efficacy in alleviating the side effects in both cases of treatment. The results showed that Meriva® may reduce the burden of side effects related to chemo- and radio-therapy, which indicates that the application of various preparations of curcumin as a support agent for cancer treatment is worthy of systematic research in larger sample clinical trials [208].

### 4.3.2 Improvement of curcumin specificity

#### 4.3.2.1 Curcumin sensitivity and MSI status

Improving curcumin bioavailability should not only promote absorption as describe above, but also depend on tumor-specific and patient-specific factors. In the present study, most CRC cell lines with the molecular type of MSS or high IκB level were more sensitive to curcumin, but 9/11 CRC cell lines with the characteristics of MSI and low IκB level were strongly resistant to curcumin.

Similarly, Shakibaei et al. [157] reported that curcumin combined with 5-FU could impact MMR-deficient CRC cells, however was more sensitive to MMR-proficient CRC cells. Moreover, in another study of combinatorial treatment *in vitro*, two human CRC cell lines including Caco-2

characterized by MSS and D.L. Dexter 1 (DLD-1) characterized by MSI [211], were used for evaluating the sensitivity to combination of curcumin and resveratrol. The results showed that $IC_{50}$ values of curcumin and resveratrol were 71.8μM (20.5μM curcumin + 51.3μM resveratrol) for the DLD-1 cell line, but a relatively lower value of 66.21μM (18.9μM curcumin + 47.3μM resveratrol) for the Caco-2 cell line, respectively [212].

However, Majumdar et al. reported an opposite result, with a better effect shown in HCT116 (MSI) after treatment with curcumin and resveratrol, but not in HT29 characterized as MSS [213]. Moreover, Jiang et al. presented that the MMR system is strongly associated with the cellular response to curcumin in human hereditary non-polyposis CRC [214]. A heightened cytotoxic response to curcumin was observed in MMR-deficient CRC cell lines (MLH1-deficient cell lines: HCT116+ch2 and RKO; MSH2-deficient CRC cell line: Hec59) compared with matched MMR-proficient cell lines (MLH-proficient CRC cell lines: HCT116+ch3 and RKO+$MLH1^{+}$; MSH2-proficient CRC cell line: Hec59 +ch2). As a result, these findings proposed that curcumin might be more specific for MSI cancers. In addition, the MSI-negative cancers may show a good response to the combination treatment of curcumin and Chk1 inhibitors [214]. In 2016, they reported that CRC cell lines harboring mutations in MMR exhibited higher sensitivity to curcumin as a result of durable unrepaired DNA damage induced by curcumin. Their study further presented that GADD45α modulates curcumin sensitivity through a mechanism involving c-Abl tyrosine kinase-mediated activation of c-Jun N-terminal kinase in a MMR-dependent manner [215, 216].

Moreover, Wei et al. [217] displayed a potent apoptosis-inducing ability of curcumin on MMR-deficient CRC cell lines (HCT116, LoVo, SW48, and HCT15). However, this study lacked

proper control groups with the feature of sufficient MMR, thus, this cannot conclude higher sensitivity in MMR-deficient CRC cell lines without comparison.

In an exploration of the underlying mechanism, Aussem et al. showed that nutritional Nrf2 activators (such as sulforaphane, curcumin, quercetin, resveratrol) have the potential to activate the cellular anti-oxidative system. And patients with lynch syndrome alteration have a high risk of cellular carcinogenesis caused by oxidative stress. Therefore, this study hypothesized that nutritional Nrf2 activators may have the potential to reduce the risk of cancer in lynch patients by modulating oxidative stress and inflammation [218].

Firstly, this present data is supportive for the point as far as curcumin could decrease cell viability of all 32 CRC cell lines with the characteristics of not only MSI, but also MSS. Also, a higher cell death was observed in both MSI and MSS cell lines at the high treatment dose of 20µM. However, constantly and repeatedly, the majority of MSS CRC cell lines were found more susceptible to curcumin, whereas a response to curcumin was observed for most MSI CRC cell lines only for very high concentrations of 20µM or 25µM. The $IC_{50}$ for two MSS CRC cell lines (HROC18 and HROC357) were as low as 6.69µM and 6.97µM, respectively. However, close to 3-fold higher $IC_{50}$ of curcumin were found in the MSI CRC cell lines HROC24 and HROC285. In order to exclude the potential bias of individuality, especially in the light of the above mentioned studies with the opposite findings that curcumin-sensitive CRC cell lines were mostly MMR deficient, analyzing a larger number of CRC cell lines (n = 32) from patients or PDX models where clinical and tumor information was more available [138]. Of note: all cell lines used in the present study are below

passage number 50, and thus can still be considered as suited to closely mirror the clinical behavior and biology of the original patients' tumors.

#### 4.3.2.2 Curcumin sensitivity and IκB level in CRC cells

Mechanistically, the NF-κB signaling pathway is well known to represent the classical pathway responsible for the anti-cancer action of curcumin. This present work could identify the NF-κB inhibitor, IκB as a potent biomarker for predicting curcumin-sensitivity of CRC cell lines.

A study from Sandur et al. [130] described that most patients becoming resistant to radiation therapy due to an activation of the NF-κB pathway. Thus, to overcome this issue, one possible way is to stimulate the inhibitors of these pathways, accordingly, making these tumors sensitive to radiotherapy again. They could prove this expectation that albeit radiation activated the NF-κB signaling pathway and thus led to resistance of therapy, curcumin treatment could reverse this radio-resistance by suppressing the radiation-induced NF-κB activation. In more detail, the elevation of NF-κB signaling induced by radiotherapy was mediated by stimulating Akt phosphorylation, inhibitor of κB kinase (IKK) activation as well as IκBα phosphorylation. Moreover, curcumin also inhibited NF-κB-regulated gene products, such as Bcl-2, Bcl-x(L), inhibitor of apoptosis protein-2, cyclooxygenase-2 and cyclin D1.

Similarly, Shakibaei et al. [129] also reported that curcumin could reverse 5-FU treatment resistance. Curcumin enhanced 5-FU-induced expression of pro-apoptosis proteins (caspase-3, caspase-8, caspase-9, PARP and Bax), and on the other hand, weakened the anti-apoptosis proteins such as, Bcl-x(L) and down-regulated cell proliferation proteins as for example cyclin D1. And this

down-regulation of the signaling by curcumin is mainly through suppressing the IKK activation and IκB alpha phosphorylation [129].

De-regulation of NF-κB as well as inhibition of its downstream targets has been proposed as an important targetable mechanism of CRC resistance to therapy [219]. IκB alpha was considered as a key protein to reverse the chemotherapy induced up-regulated NF-κB signaling pathway. This was explained by curcumin inhibiting the oxaliplatin-induced activation of NF-κB and decreasing the expression of NF-κB anti-apoptotic and pro-proliferative gene products. Different gene expression patterns were shown in oxaliplatin-resistant and -sensitive CRC cell lines after combination treatment of curcumin and oxaliplatin. As compared to oxaliplatin-sensitive CRC cells, curcumin more extensively inhibited the NF-κB level in oxaliplatin-resistant cell line [128].

Similar results were also obtained in the present study, as I measured increasing IκB levels in response to curcumin treatment in six CRC cell lines including three curcumin-sensitive and three -resistant ones, respectively. Compared to curcumin-resistant CRC cell lines, the curcumin-sensitive CRC cell lines showed very higher IκB levels at the same curcumin concentration. Taken together, experimental evidence has proven that curcumin treatment is associated with inhibition of NF-κB, activation of IκB alpha, suppression of IκB alpha phosphorylation as well as down-regulation of NF-κB related anti-apoptotic and cell proliferation proteins [220-222]. In the present study, IκB alpha was identified as an important biomarker to predict the curcumin sensitivity of CRC cell lines. In the following, a detailed overview was given on this classical pathway.

NF-κB transcription factors consist of five homologous subunits including RelA/p65, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2, which dimerize with the IκBs and are retained in the cytoplasm [223, 224]. The IKK complex is immediately upstream of the IκB-bound NF-κB dimer. It consists of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ/NEMO) [225, 226]. When the IKK complex is activated through several cell stimulation pathways, IκB is phosphorylated and thereby targeted for ubiquitination and degradation by the 26S proteosome. Furthermore, the released NF-κB travels to the nucleus and participates in transcriptional programs [227].

Briefly, there are two NF-κB activation signaling pathways named canonical and non-canonical pathway [223] (Figure 21). The non-canonical NF-κB activation is associated with lymphoid organogenesis via activation of NF-κB-inducing kinase (NIK) [228-230], whereas the canonical NF-κB activation depends on IKK activation, nuclear localization of RelA/P50 dimers, IκB phosphorylation or degradation. And it has been proved that high RelA/p65 expression is correlated with increased expression of NF-κB target genes. Both of them, but even more the classical NF-κB activation pathway are crucial for progression of carcinomas [230-236].

In sum, enhanced degradation of IκB directly contributes to the activation of NF-κB and impacts the tumor cell proliferation, apoptosis, cell cycle and migration as well as invasion. In addition, curcumin has been found to mediate its anti-tumor activity by down-regulation of NF-κB via IκB activation. Thus, IκB as biomarker can likely predict the curcumin sensitivity of CRC cell lines. A significant correlation between $IC_{50}$ of curcumin and the IκB level was presented in CRC cell lines measured before any treatment. It is worthy to pronounce that the cell viability is strongly associated with the IκB level after curcumin treatment.

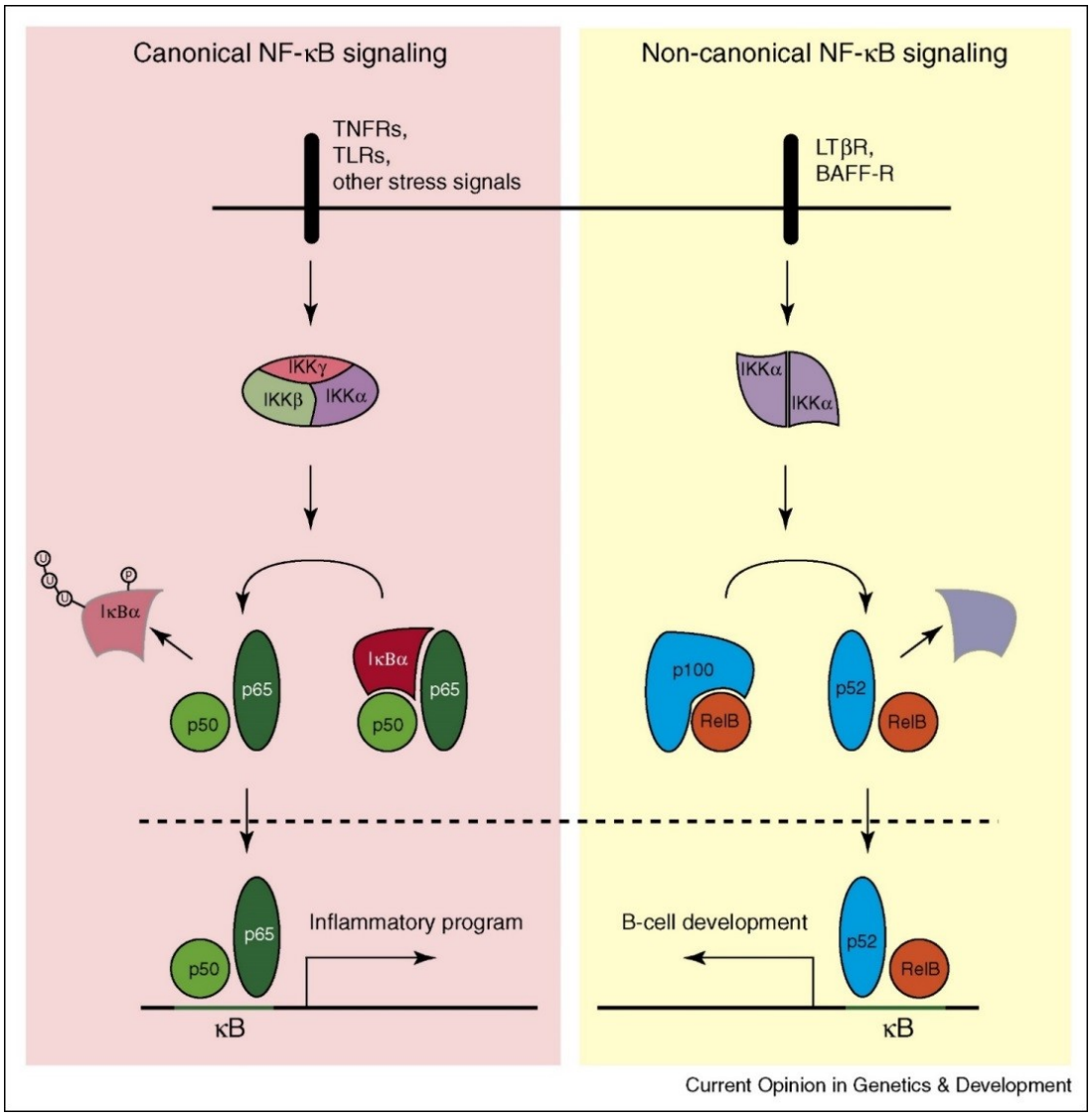



Figure 21. The two main signaling pathways of NF-κB activation [226].

In addition, a growing number of studies showed that NF-κB activation is correlated with interleukin-6 (IL-6) [105, 237-241] and (interleukin-8) IL-8 [241-244] (Figure 22). Pre-treatment with curcumin effectively suppressed NF-κB activation coinciding with a decreased amount of IL-6 and IL-8, which also demonstrated the key role of the NF-κB pathway in augmenting expression of these cytokines [238]. In continuation, Das and colleague [245]s reported that the expression of IL-6

mRNA as well as protein levels were significantly down-regulated by curcumin in *vivo*. They additionally observed that curcumin reduced the NF-κB binding elements of TNF-α and IL-6 promoters. This study thus suggested that it might be an interesting long-term effect of curcumin to reduce cancer progression via down-regulation of TNF-α and IL-6. However, since I did not measure IL-6 and IL-8 secretion of the CRC cell lines included into this study, they used a potential additional biomarker for curcumin sensitivity can only be speculated on.

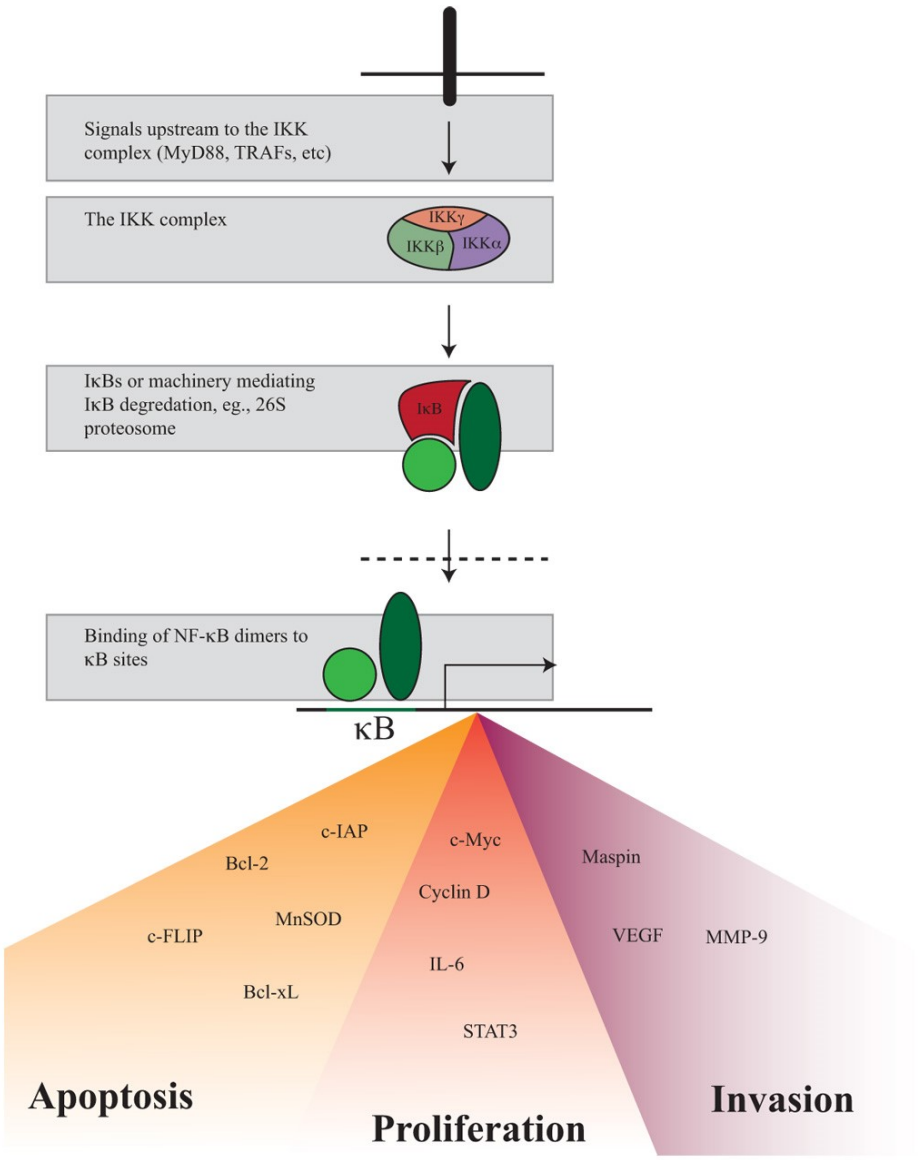



Figure 22. Targeting potential NFκB contributors to carcinogenesis [226].

### 4.3.3 MSI status and IκB level in CRC cells

In the present study, I defined two possible biomarkers (MSI status and IκB level in CRC cells) which are predicting curcumin sensitivity of CRC cell lines. Surprisingly, a higher level of IκB was seen in MSS, but not in MSI CRC cells. When combining the MSI status with the IκB level as biomarkers, a significant and strong prediction ability could be demonstrated. The present study is the first to report on the curcumin sensitivity of mostly MSS CRC cells with higher IκB levels, but resistance in mostly MSI CRC cells with lower IκB level. These findings are somehow opposing the data from some previous studies [213, 214, 217, 246].

However, since my data have been obtained with a relatively high number of low-passaged cell lines, even allowing for statistical comparison, they can be considered as relevant. Concerning the data from Wei [217], Majumdar [213] Jiang [214] Fonseca [246] and colleagues, one can only speculate that a part of the opposite finding might best be explained by the fact that only a limited number of classical CRC cell lines has been analyzed and moreover, the focus of their study was not curcumin but several drugs.

In a general analysis of the Raf-1 Kinase Inhibitor Protein (RKIP), Koelzer et al. [247] found that RKIP loss significantly predicted for tumor metastasis, vascular invasion, tumor budding as well as invasive border configuration. Deficiency of RKIP caused greater NF-κB activation and was more frequently observed in MMR-deficient cancers; however, was not linked to mutations in K-RAS and B-Raf [247]. It is thus tempting to speculate, that on the one hand, the curcumin sensitive MMR proficient HROC cell lines eventually have a high expression of RKIP and that on the other hand, the

curcumin resistant MMR deficient cell lines might have lost or reduced RKIP expression. However, this needs to be analyzed in another study.

Moreover, the alkylating agent temozolomide influences malignant glioma as a result of cellular cytotoxicity by forming O (6)-methylguanine adducts. And effectiveness of temozolomide is consistent with inhibition of NF-κB activity, also inhibits NF-κB activation by TNF as well as other inducers (lipopolysaccharide, doxorubicin, and phorbol 12-myristate 13-acetate). It is important to mention that the ability of temozolomide for inhibition of NF-κB activation is in the presence of a functioning MMR system. Whereby, their work showed that temozolomide leads to inhibition of NF-κB activity and explained a correlation between MMR processing of alkylator-induced DNA damage and cell apoptosis [248]. Based on these data, and in line with the RKIP-loss data discussed above [247] high NF-κB activation seems to be associated with a deficient MMR system. These findings are also matching my results concerning NF-κB activation mostly in MMR deficient HROC cell lines. In addition, Chen et al. [249] evaluated the effect of curcumin on MMR proteins (hMSH2 and hMLH1) after ultraviolet (UV) irradiation of leukemic cells. The expression of hMSH2 and hMLH1 genes was not influenced by UV irradiation alone, but significantly increased when UV irradiation was combined with a curcumin treatment. Moreover, this combination treatment also induced cell apoptosis. The latter can best be explained by the dual role of the MMR system – either repair mismatched DNA or, when this fails, induce cellular apoptosis [249]. These data might also explain why curcumin acted more effectively on MMR proficient HROC cell lines.

Opposite to these findings, Fonseca and colleagues [246] evaluated the correlation between MMR genes (hMSH2 and hMSH6) expression and NF-κB in 28 samples of patients with oral squamous

carcinoma [246]. There was a significant correlation between the expression of NF-κB and hMSH2 ($r_s$ = 0.570, $P$ < 0.001) as well as hMSH6 ($r_s$ = 0.658, $P$ < 0.001). They concluded that activating the transcription of NF-κB (inflammatory principle) in turn activates the DNA repair system (hMSH2 and hMSH6); a hypothesis which at least could explain the involvement of the inflammatory component in the MMR system. Thus, modulating the expression of NF-κB may be essential for the control of neoplasia, ageing of cells, and above all cell survival in important tissues and organs. Alcohol consumption and smoking have a remarkable influence on the DNA repair system in oral squamous cell carcinoma [246]. However, the 28 samples have not been stratified into MMR proficient or deficient.

In sum, data of different studies indicated that the response to curcumin of cancer, and especially CRC cells, is mediated by inhibition of NF-κB by IκB, and seems to additionally be linked to the MMR system; albeit the exact mode of action still needs to be determined.

# 5. Conclusions

CRC still is a big burden worldwide with a still unsatisfying survival rate, especially for patients having distant metastases. Currently, chemotherapeutic and also novel drugs have significantly improved patients' survival, but there are two major issues of side effects and the development of drug resistance. Therefore, natural products like curcumin with proven antitumoral action are under research as promising candidates to contribute to anti-tumor treatment.

This present study could demonstrate that curcumin can inhibit CRC cell viability, induce cell apoptosis, suppress cell migration and invasion as well as induce cell cycle arrest at the G2/M stage *in vitro*. The 32 CRC cell lines analyzed respond to curcumin to a different extent. The focus was primarily to identify the subset of cell lines showing high curcumin sensitivity, to unravel common features of the responding cell lines in order to identify possible biomarkers which could subsequently be used to predict response to curcumin. Most curcumin-sensitive CRC cell lines have the following characteristics: molecular type of MSS and/or higher IκB expression level of the cells before any curcumin treatment. Whereas the curcumin-resistant CRC cell lines were MSI and had low cellular IκB levels. The predictive ability of this biomarker combination was statistically significant with a predictive power of around 72% in a classical ROC/AUC analysis.

In sum, these data support the ongoing activities to improve the bioavailability of curcumin since its clinical application is limited until the bioavailability is largely improved. With the help of a large panel of patient-derived low passaged CRC cell models with known sensitivity and predictive biomarker expression, novel curcumin-derivate candidate drugs can easily be analyzed. Since the

HROC model collection also includes a high number of matching PDX *in vivo* models, the complete preclinical drug testing can even be envisioned.

With the data obtained so far, I suggest that MSS patients with high IκB expression level can be expected to benefit from a clinical treatment by improved curcumin versions. Further studies should also analyze the combination of new curcumin versions with classical chemotherapeutics.

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# 7. Appendix

## 7.1 Abbreviations

| Abbreviation | Meaning |
|---|---|
| 15-PGDH | 15-prostaglandin dehydrogenase |
| AUC | Area under the ROC curve |
| BDMC | Bisdemethoxycurcumin |
| CC | Colon cancer |
| CIMP | CpG island methylator phenotype |
| CIN | Chromosomal instability |
| COX-2 | Cyclooxygenase 2 |
| CRC | Colorectal cancer |
| CSC | Cancer stem cell |
| DLD-1 | D.L. Dexter 1 |
| DMC | Demethoxycurcumin |
| EGFR | Epidermal growth factor receptor |
| Egr-1 | Early growth response protein 1 |
| FOLFIRI | Fluorouracil combined with irinotecan |
| FOLFOX | Fluorouracil combined with oxaliplatin |
| FOLFOXIRI | Combinations of fluorouracil, oxaliplatin and irinotecan |
| IKK | Inhibitor of κB kinase |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IκB | Inhibitors of NF-kappa-B |
| MAPK | Mitogen-activated protein kinase |
| MAPK | Mitogen-activated protein kinase |

| | |
|---|---|
| mCRC | Metastatic colorectal cancer |
| MLH1 | MutL homolog 1 |
| MMR | Mismatch repair |
| MSH2 | MutS homolog 2 |
| MSH6 | MutS homolog 6 |
| MSI | Microsatellite instability |
| MSS | Microsatellite stability |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NIK | NF-κB-inducing kinase |
| Nrf2 | Nuclear factor-carotenoid 2-related factor 2 |
| RC | Rectum cancer |
| RKIP | Raf-1 Kinase Inhibitor Protein |
| ROC | Receiver Operating Characteristic |
| ROS | Reactive oxygen species |
| TGF-β | Transforming growth factor-β |
| UICC | Union Internationale Contre le Cancer |

## 7.2 Figure list

## 7.3 Table list

# 8. Thesis statements

- Curcumin's clinical application has been found to be restricted by its poor bioavailability. However, novel drug formulations currently under research suggest that a "revival" of this very old natural compound seems possible also for oncological purposes.
- Curcumin inhibits cell proliferation, accelerates cell apoptosis, induces cell cycle arrest in G2/M stage as well as suppresses cell migration and invasion in colorectal cancer (CRC) cell lines.
- The sensitivities to curcumin of the 32 CRC patient-derived cell lines tested are highly individual.
- Mutations of the frequently mutated genes in CRC: APC, K-Ras, B-Raf, PIK3CA, TP53 and ATM were found to be not related to curcumin sensitivity or resistance in CRC cells.
- Most microsatellite instable (MSI) CRC cell lines are resistant to curcumin, whereas most microsatellite stable (MSS) CRC cell lines are sensitive to curcumin.
- Curcumin-sensitive cell lines express significantly higher levels of inhibitors of NF-kappa-B (IκB), while curcumin-resistant cell lines frequently express low IκB levels. IκB levels dramatically rise with increasing concentrations of curcumin.
- There is a significant difference between the IκB expression level in MSS (high) compared to MSI (low) CRC cell lines.
- The combined biomarkers microsatellite status and IκB expression level predict the curcumin sensitivity of a CRC cell line to 72%.
- When considering that MSS CRC cases hardly benefit from the novel immunotherapeutic treatment options, the fact that they are more sensitive to curcumin is of potential clinical relevance – especially for studies of novel curcumin drug formulations.

# 9. Eidesstattliche Versicherung

Ich versichere hiermit, dass ich die vorliegende Arbeit mit dem Thema: “Identification of the microsatellite stability status and inhibitor of NF-kappa-B expression levels as biomarkers to predict sensitivity to pharmaceutical curcumin in colorectal cancer cells” unabhängig verfasst habe.

Ich erkläre an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel erstellt habe. Die aus anderer Literatur verwendeten Inhalte sind als solche kenntlich gemacht. Ich versichere, dass ich für die inhaltliche Erstellung der vorliegenden Arbeit nicht die entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen habe.

Ich erkläre hiermit weiterhin, dass ich meine wissenschaftlichen Arbeiten nach den Prinzipien der guten wissenschaftlichen Praxis gemäß der gültigen „Satzung der Universität Rostock zur Sicherung guter wissenschaftlicher Praxis“ angefertigt habe.

Rostock,

| | |
|---|---|
| | Lili Lu |
| 03/04/2021 | Lili Lu |
| Abgabedatum | Vollständige Unterschrift |

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# 11. Curriculum Vitae

## Personal Details

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## Education

2018 - now Molecular Oncology and Immunotherapy, University of Rostock, Germany

2015 - 2018 Master of Medicine in Epidemiology, Dalian Medical University, China

2010 - 2015 Bachelor of Preventive Medicine, Inner Mongolia Medical University, China

## Publication

### First Author Publications

1. **Lu L**, Shang Y, Mullins CS, Zhang X, Linnebacher M. Epidemiologic trends and prognostic risk factors of patients with pancreatic neuroendocrine neoplasms in the US: an updated population-based study. Future Oncol 2021; 17(5):549-563.

2. **Lu L**, Ma L, Zhang X, Susanne Mullins C, Linnebacher M. Analyzing non-cancer causes of death of colorectal carcinoma patients in the US population for the years 2000-2016. Cancer Med 2021; 10(8): 2740-2751.

3. **Lu L**[#], Shang Y[#], Zechner D, Mullins CS, Linnebacher M, Zhang X, Gong P. Development and validation of a score for screening suicide of patients with neuroendocrine neoplasms. Front Psychiatry 2021; 12:638152.

4. **Lu L**, Zhang X, Tang G, Shang Y, Liu P, Wei Y, Gong P, Ma L. Pancreaticoduodenectomy is justified in a subset of elderly patients with pancreatic ductal adenocarcinoma: A population-based retrospective cohort study of 4,283 patients. Int J Surg 2018; 53:262-268.

5. Shang Y[#], Zhang X[#], **Lu L**[#], Jiang K, Krohn M, Matschos S, Mullins CS, Vollmar B, Zechner D, Gong P, Linnebacher M. Pharmaceutical immunoglobulin G impairs anti-carcinoma activity of oxaliplatin in colon cancer cells. Br J Cancer 2021; 124(8): 1411-1420.

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**Co-author Publications**

1. Zhang X, Song J, Liu P, Mazid MA, **Lu L**, Shang Y, Wei Y, Gong P, Ma L. A modified M-stage classification based on the metastatic patterns of pancreatic neuroendocrine neoplasms: a population-based study. BMC Endocr Disord. 2018; 18(1):73.

2. Liu P, Zhang X, Shang Y, **Lu L**, Cao F, Sun M, Tang Z, Vollmar B, Gong P. Lymph node ratio, but not the total number of examined lymph nodes or lymph node metastasis, is a predictor of overall survival for pancreatic neuroendocrine neoplasms after surgical resection. Oncotarget. 2017; 8(51): 89245-89255.

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1. **Lu L**, Zhang X, Ma L, et al. Prediction effect of the total number of examined lymph node on N staging and survival in pancreatic neuroendocrine neoplasms. 20th Annual Meeting Chinese Society of Clinical Oncology (CSCO), 2017, Xiamen, China (poster).
2. Song J, Zhang X, Wang Zhao, **Lu L**, et al. The lymph node ratio as an indicator of the diagnosis and prognosis of liver metastases in pancreatic neuroendocrine neoplasms. 20th Annual Meeting Chinese Society of Clinical Oncology (CSCO), 2017, Xiamen, China.

## Honors and Awards

**2019**

China Scholarship Council (grant number: 201908080127)

**2015 - 2018**

Outstanding graduate of Dalian City

Excellent Student Leader of Dalian Medical University

Scholarship of Dalian Medical University

Outstanding Student of Dalian Medical University

**2010 - 2015**

Outstanding graduate of Inner Mongolia Autonomous Region

Outstanding graduate of Inner Mongolia Medical University

Scholarship of Inner Mongolia Medical University

Excellent Student Leader of Inner Mongolia Autonomous Region

Outstanding young volunteers of Inner Mongolia Medical University

Individual Scholarship of Inner Mongolia Medical University

Outstanding Volunteer of summer social practice of the whole region

Outstanding Individual of summer social practice of Inner Mongolia Medical University

Excellent work award of Inner Mongolia Medical University

Single Scholarship of Inner Mongolia Medical University

## Signature

Lili Lu

Rostock, 03/04/2021 Lili Lu

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