ISSN: 03875547
Volume 44, Issue 05, October, 2021

Effect of Adoptive T cell Therapy on Colon Cancer Growth and Angiogenesis: an in vitro and in vivo study

Mirna Bou Hamdan[1], Michel Massaad[2], Julnar Usta[3], Jamila Borjac[4*]

Department of Biological Sciences, Faculty of Science, Beirut Arab University, Debbieh, Lebanon; Medical Laboratory Sciences Program, Division of Health Professions, Faculty of Health Sciences, American University of Beirut, Beirut, Lebanon[1]

Department of Experimental Pathology, Immunology, and Microbiology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon; Department of Pediatrics and Adolescent Medicine, American University of Beirut Medical Center, Beirut, Lebanon; Center for Infectious Diseases Research, American University of Beirut, Beirut, Lebanon[2]

Department of Biochemistry and Molecular Genetics, Faculty of Medicine, American University of Beirut, Beirut-Lebanon[3]

Department of Biological Sciences, Faculty of Science, Beirut Arab University, Debbieh, Lebanon[4*]

Corresponding author: 4*

Keywords:
Colon cancer, Adoptive Immunotherapy, Chemotherapy, Angiogenesis, Oncogenes

ABSTRACT

Adoptive Cell Transfer (ACT) is a new type of immunotherapy that relies on boosting the ability of natural T cells to kill tumor cells. The aim of this study is to determine the effect of ACT and/or Sorafenib on colon cancer growth in vitro and on angiogenesis in vivo. HCT 116 and p53-/- cells were cultured in vitro and treated with different concentrations of either CD8 T cells isolated the PBMC of 2 healthy individuals or Sorafenib. The effect of both treatments on both cell lines was assessed using MTT. Colon cancer was induced using DMH injections in Balb/c. The mRNAs from the homogenized colonic tissues of chemotherapeutic and immunotherapeutic-treated mice were transcribed and quantified using RT-PCR. Results show that the IC50 of Sorafenib against HCT 116 and HCT 116 p53-/- cells was 50 and 35 µM, respectively. The IC50 of CD8 T cells in both cell lines was 10:1. In vivo, Sorafenib and/ or ACT significantly decreased the expressions of c-MYC (12-16 folds, p value <0.01), cyclin-D1 (18 folds, p value <0.001), PDGF-β (5-7 folds, p value <0.05), PDGF α (2-4 folds, p value <0.01) and β (3-6 folds, p value <0.001) receptors as compared to the untreated mice. Treatments significantly increased the expressions of CDC-4 by around 3 folds (p value <0.05) and non-significantly elevated the expressions of caspase-9 by 0.5 folds (p value >0.05) as compared to untreated mice. ACT can be used as a substituent or an adjunct to chemotherapy in colon cancer treatment.

1. INTRODUCTION

Colon cancer is one of the most common types of cancers worldwide affecting both male and females [1]. There are several environmental and genetic factors implicated in colon cancer development and progression [2]. Deaths resulting from this type of cancer were mainly linked to the development of resistance to various treatment methods [3]. Despite the work that was done to understand the different aspects of colon cancer in relation to disease progression, diagnosis, and treatment, adverse health results were still observed among patients especially those treated at progressive stages of the disease [4]. There are several pathways implicated in colon cancer progression [4]. The platelet-derived growth factor/platelet-derived growth factor receptor (PDGF/PDGFR) pathway is a significant signaling pathway involved in angiogenesis using surrounding vasculatures, drug resistance, and metastasis [5]. This pathway is critical for the normal development of mesenchymal cells. PDGF affects the proliferation, survival, and tissue metastasis of cancer cells [5]. Two tyrosine kinase receptors, PDGFR alpha (PDGFRα) and PDGFR beta (PDGFRβ), and five ligands (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD) are involved in the PDGF/PDGFR angiogenic signaling pathway. This pathway play a role in malignant transformation through the recruitment of various types of cells such as stromal cells to the tumor environment, thus aiding in enhancing tumor growth and activating tumor angiogenesis and metastasis. Since PDGF signaling can also lead to an increase in interstitial fluid pressure (IFP) in most solid tumors, several chemotherapeutic treatments included the inhibition of PDGF signaling to increase the efficacy of cancer cells elimination or inhibition [5]. CDC-4 (also known as FBXW7), a tumor suppressor gene located on human chromosome 4q, encodes the substrate recognition components of SKP1-Cullin1-F-box protein ubiquitin E3 ligase complexes [2, 6]. The encoded complexes play a vital role in negatively regulating the intracellular amount of various key oncogenic proteins. Therefore, the loss of CDC-4 function results in oncogenesis and colon cancer progression due to the accumulation of its corresponding substrates [2]. Caspase-9 is the main member of caspase family in inducing endogenous apoptotic pathway [7]. It was evidenced that caspase-9 mediated apoptosis might reflect the susceptibility of cancer cells to chemotherapy drugs since caspase-9 suppression is one of the mechanisms followed by cancer cells to escape apoptosis [8]. c-MYC is a proto-oncogene involved in cell cycle, survival, protein synthesis, and cell metabolism. It was proposed that c-MYC gene is usually over-expressed in both sporadic and colitis-associated colon adenocarcinomas. c-MYC is associated with colon cancer progression and poor prognosis of colon cancer patients [9]. Cyclin-D1 is a proto-oncogene and a major regulator of cell proliferation and generation of tumor cells. The expression of cyclin-D1 is over-regulated in colon cancer cells [10].

Various types of immunotherapeutic treatments were used to eliminate cancer cells [11]. CD8 T cells are immune cells that play a critical role in cancer elimination and immunoediting in our body [12]. Adoptive Cell Transfer (ACT) is a new type of immunotherapeutic treatments that relies on boosting the natural ability of CD8 T cells to fight cancer. The latter is performed through isolating cancer-specific CD8 T cells in vitro and then re-injecting them to treat the same type of cancer in vivo [13]. It was proposed that naturally occurring cytotoxic CD8+ T cells can eliminate metastatic gastrointestinal cancers in vivo despite the limitations due to the low number of tumor specific CD8 T cells [14]. Moreover, chemotherapy prior to CD8 T cell therapy was able to boost the effectiveness of anticancer CD8 T cells in vivo; the latter suggests that immunotherapy may be used as an adjunct to chemotherapy in cancer treatment [15]. Sorafenib is a tyrosine kinase inhibitor used in chemotherapeutic treatment of various types of cancers including colon cancer. The mechanism of action of this drug relies on its ability to kill tumor cells via inhibiting various angiogenic pathways involved in cancer progression and metastasis, such as mitogen- activated protein kinase (MAPK) signaling pathways, vascular endothelial growth factor receptor (VEGFR), Platelet-derived growth factor receptor (PDGFR) and Raf family kinases [16]. The aim of this study is to determine the effect of ACT and/or Sorafenib on inhibiting the growth of colon cancer cell lines in vitro and angiogenesis

in vivo. To our knowledge, this is the first study to target the effect of ACT on the expression of genes associated with PDGF/PDGFR pathway in colon cancer in vivo and to compare the effect of CD8 T cells to Sorafenib on inhibiting the growth of colon cancer cell lines in vitro.

2. Methods

2.1 In Vivo

2.1.1 Colon Cancer Induction

A total of 36 female Balb/c mice (6 weeks old) were used in this study (6 groups; group 1 includes the normal control). The 5 other groups received intraperitoneal injections with 1, 2-dimethylhydrazine (ACROS OrganicsTM, Thermo Fisher Scientific, USA) at 20 mg/kg body weight over a period of 12 consecutive weeks to induce colon cancer in vivo [23]. One week after the last DMH injection, 6 mice were fasted overnight, then sacrificed and their spleens and lymph nodes were excised to isolate CD8+ T cells. After treating and dissecting all mice groups, the entire large intestines were excised from the six groups to determine the effect of the treatment of gene expression.

2.1.2 CD8 T cell Culture

Spleens in addition to inguinal, mesenteric and axillary lymph nodes were excised from the DMH injected Balb/c mice. The single-cell suspensions isolated from these tissues were pooled, filtered using a 40 µm cell strainer (Fisher), and counted. Splenic and lymph node CD8+ T cells were isolated and enriched by negative selection using Mojosort Mouse CD8 T cell Isolation Kit (cat no 480035, BioLegend, USA), Mojosort Buffer 5x (cat no 480017, BioLegend, USA) and Mojosort Magnet (cat no 480020, BioLegend, USA) according to manufacturer's protocol. The purity of cells was assessed via Flow cytometry using anti-CD8-APC (clone 53-6.7, BioLegend, USA), anti-CD4-FITC (clone GK 1.5, BioLegend, USA), and anti-CD19- PerCP/Cyanine5.5 (clone 1D3, BioLegend, USA). CD8 T cells were plated into 6-well plates (Thermo Fisher Scientific) coated with 0.5 µg/mL purified anti-mouse CD3 (cat no 100301, BioLegend, USA) and 5 µg/mL Purified anti-mouse CD28 (cat no 102101, BioLegend, USA) in PBS (5 mL/well) overnight at 4oC. The CD8+ T cells were cultured (5 x 106 per well) for 3 consecutive days in presence of 0.5 ng/mL of Recombinant Mouse IL-7 (cat no 577804, BioLegend, USA) and 30 U/mL of Recombinant Mouse IL-2 (cat no 714604, BioLegend, USA). On day 3, cells were harvested from the wells, resuspended in the medium, subcultured in a 6-well plate at 1x106 cells/5 ml/well in the presence of fresh medium containing all supplements and IL-2 and IL-7; the latter was repeated twice. On day four, cells were harvested and used for adoptive transfer 2 weeks following the last DMH injection as shown in Table I.

Table I. Experimental Design. All mice treatments were given 2 weeks after the final DMH injection and continued for 5 consecutive days.

Group A	Normal control (no injections).
Group B	DMH-injected Group: DMH intraperitoneal injections (20 mg/Kg) once per week over 12 weeks. The tumor specific CD8 T cells were obtained from this group.
Group C	Vehicle-treated Group: oral DMSO by gavage.
Group D	Chemotherapeutic-treated Group: oral Sorafenib Tosylate (cat no 1100200013; Eton Bioscience Inc) treatment (30 mg/kg) by gavage.

Group E	CD8 T cell- treated Group: CD8 T cell tail IV injections at 10 x 10^6 cells per mouse.
Group F	Sorafenib + CD8 T cell-Treated Group: received oral Sorafenib Tosylate treatment at 30 mg/kg in combination with CD8 T cell IV injections at 10 x 10^6 cells per mouse.

2.1.3 Quantification of signaling gene expressions by RT-PCR

Total RNA was extracted from colon homogenates using the Direct-zol RNA Miniprep (cat no R2051, Zymo Research, USA) according to the manufacturer's protocol. The purity and integrity of the eluted RNA samples were checked via gel electrophoresis and quantified using DeNovix (Blue) DS-11 Spectrophotometer (DNA-RNA Quantification) at 260 nm absorbance. RNA was transcribed using the QuantiTect® Reverse Transcription Kit (cat no 205311, QIAGEN, USA) according to manufacturer's protocol. The cDNA obtained was stored at –70 °C for later use. The primers used to quantify the expression levels of CDC-4 (Forward (F): 5'-ACCAGCTCTCCTCTCCATTCT-3' and Reverse (R): 5'-CAACTTCTCTGGTCCGCTCC-3' [37]), Caspase-9 (F: 5'-AGTTCCCGGGTGCTGTCTAT-3', R: 5'-GCCATGGTCTTTCTGCTCAC-3'[38], c-MYC (F: 5'-TAGTGCTGCATGAGGAGACA-3'; R: 5'-GGTTTGCCTCTTCTCCACAG-3'[39]), cyclin-D1 (F: 5'-GGCACCTGGATTGTTCTGTT-3'; R: 5'-CAGCTTGCTAGGGAACTTGG-3' [39]), PDGF-beta (F: 5'-TGGAGTCGAGTCGGAAAGCT-3', R: 5'-GAAGTTGGCATTGGTGCGAT-3' [40]), PDGF-β Receptor (F: 5'-GCACCGAAACAAACACACCTT-3'; R: 5'-ATGTAACCACCGTCGCTCTC-3'[40]), and PDGF-α Receptor (F: 5'-GAGACCCTCCTTCTACCACCT-3'; R: 5'-GTTGTCAGAGTCCACACGCAT-3'[40]) were purchased from Macrogen, South Korea. Gene quantification was performed by RT-PCR using 5x HOT FIREPol® EvaGreen qPCR Mix Plus (no ROX) (cat no 08-25-00001, Solis BioDyne, Estonia) according to manufacturer protocol. Each RT-PCR was performed in triplicate for yield validation. Gene expression was measured by comparative threshold cycle (Ct) method using glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as a reference gene; F:5'-TGGTGCTCAGTGTAGCCCAG-3' and R: 5'-GGACCTGACCTGCCGTCTAG-3' [41]. For each gene, the mean Ct (mCt) values were determined. The relative quantity of gene of interest expression compared to GAPDH gene was calculated applying the gene dosage ratio formula (GDR = $2^{-\Delta\Delta Ct}$) where:

$\Delta\Delta Ct$ = (mCt gene of interest − mCt GAPDH) control sample − (mCt gene of interest – mCt GAPDH) test sample.

2.2 In vitro

2.2.1 Treatment of colon cancer cell lines with CD8 T cells

Peripheral blood mononuclear cells (PBMCs) were separated from the peripheral blood of 2 healthy volunteers. PBMCs were cultured in RPMI1640 media containing 10% FBS. Following removal of non-adherent cells, Recombinant mouse GM-CSF protein (Active) (ab259385; 500 ng/ml; abcam) and IL4 (12.5 ng/ml; Biolegend) were added to induce the development of PBMCs into DCs. Colorectal cancer cell lysates (HCT 116 and HCT 116 P53-/-) and human tumor necrosis factor-α (TNFα; 50 ug, SRP3177; Sigma Aldrich) were added at day 5, and mature DCs were harvested from day 7 to day 10. The growth status of cells was observed daily using an inverted microscope. CD8 T cells were separated from PBMC of the same healthy volunteers via Mojosort Magnet (Biolegend) and cultured in vitro. Cancer lysate-loaded DCs and CD8 T cells were co-cultured in 96well plates in a 1:1 ratio. Following co-culture for 72 h, cells were counted via Trypan Blue method. CD8 T cells were induced following co-culture with DCs for 72 h. CD8 T cells (effectors) and tumor cells (targets) were co-cultured at a ratio of 5:1, 10:1 and 15:1 for 16 h in 96-well plates. The killing capacity of CD8 T cells was detected using an MTT assay at 595 nm.

2.2.2 Treatment of colon cancer cell lines with Sorafenib

Colon cancer cell lines were cultured in a 96-well plate and treated with different concentrations of Sorafenib. Moreover, the colon cancer cell lines, HCT 116 and HCT 116 p53-/-, (after reaching 80% confluency) were seeded in 96 well plate for the MTT assay for 24 hrs at 37˚C in 5% CO2 incubator. Seeded cells were treated for 24 hrs with Sorafenib at different concentrations (10-75 µM). After 24 hrs of incubation, the cells in each well were counted using hemocytometer and the light absorbance by those cells was detected using a spectrophotometer at 595 nm for the MTT assay.

2.3 Statistical Analysis

All statistical analyses were performed using Microsoft Excel and SPSS 25, and they are shown as mean with standard deviations. Statistical significance was assessed using One-way ANOVA test followed by T-test. Graphs were drawn by GraphPad prism software and statistical significance was reported with a p-value < 0.05 considered as significant.

3. FINDINGS

I. In Vivo

3.1 Effect of Sorafenib and CD8 T cell treatment on Gene expression levels

3.1.1 Effect of Sorafenib and CD8 T cell treatment on Gene expression levels of PDGF and PDGFR

The effect Sorafenib, CD8 T cell or their combination on the expression levels of the ligand PDGF-β and its α and β receptors was analyzed in all experimental groups (Figure 1). DMH (Group B) induced a significant increase in PDGF-β expression levels by 5.7 folds, p value= 0.017) compared to normal control as seen in Figure 1A. DMSO, the vehicle used to dissolve Sorafenib, induced further significant increase in CRC mice (Group C) of 7.5 folds (p value= 0.017) compared to the normal group. Treatment with Sorafenib, CD8 T cell or their combination (Groups D, E and F) induced significant decrease in PDGF-β expression levels by 5.85 folds (p value= 0.006), 5.97 folds (p value= 0.015), and 7.43 folds (p value= 0.017), respectively compared to the untreated CRC mice (Group C).

Similar results were obtained with both the α and β receptors gene expression. DMH alone (Group B) and CRC mice administered the DMSO (Group C) exhibited a significant increase in PDGF-β receptor by 10.12 folds (p value= 0.001) and 16.18 folds (p value= 0.0001) as compared to normal group (Figure 1). Sorafenib, CD8 T, or their combination (Groups D, E and F) induced a significant decrease in the expression of PDGF- β receptor by 3.92 folds (p value= 0.0001), 3.87 folds (p value= 0.0001), and 6.4 folds (p value= 0.001), respectively as compared to untreated CRC group (Group C).

Likewise, a significant increase in the expression of PDGF-α receptor was noticed among Groups B and C by 2.44 folds (p value= 0.013) and 4.23 folds (p value = 0.015) respectively as compared to normal group (Fig 1). Treatment with Sorafenib, CD8 T cell or their combination (Groups D, E and F) induced a significant decrease in PDGF-α receptor expression levels by 3.97 folds (p value= 0.004), 1.98 folds (p value= 0.005), and 4.16 folds (p value= 0.012) respectively as compared to untreated CRC group (Group C).

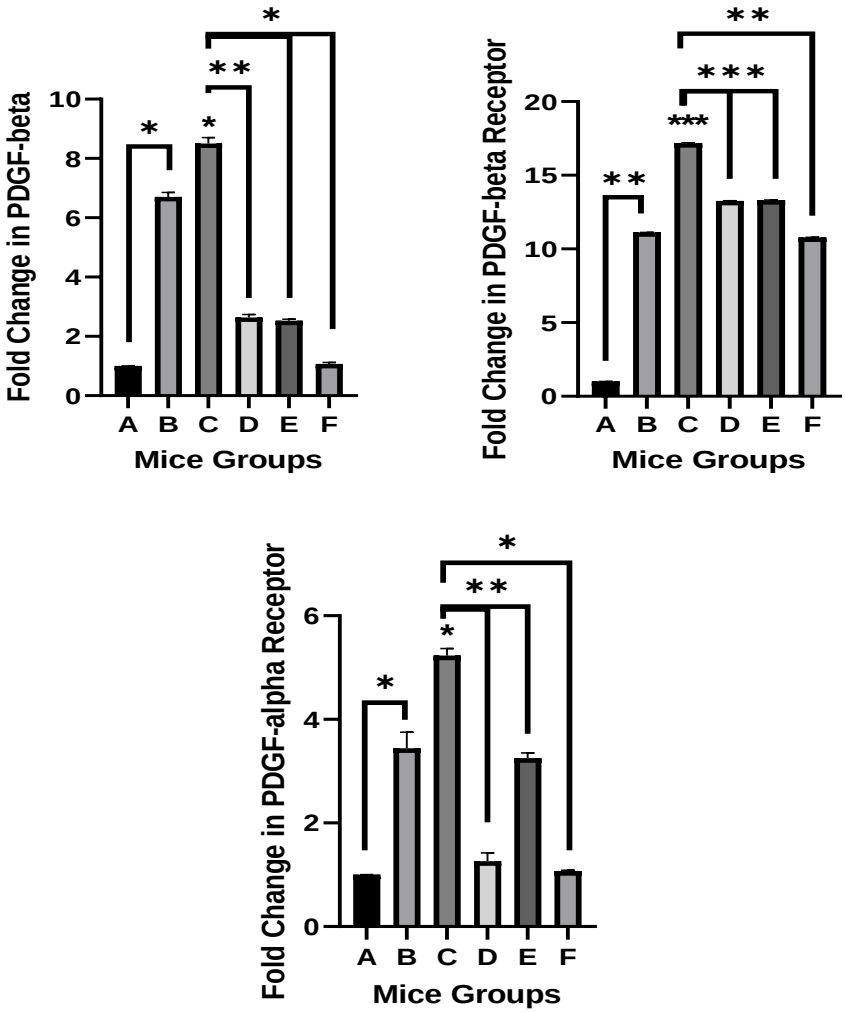


Figure 1. Effect of CD8 T cell therapy in presence or absence of Sorafenib on the expression levels of PDGF-beta, PDGF-beta receptor and PDGF-alpha receptor in colon tissues of all experimental mice groups. Expression levels of treated and control groups were normalized to their respective GAPDH. Fold expression was determined relative to the control. All bars represent mean of three determinations ± SD. (*), (**), and (***) on bars and on lines drawn upwards, that represent inter-categorical statistical significance, correspond to $p<0.05$, <0.01, and <0.001, respectively.

3.1.2 Effect of Sorafenib and CD8 T cell treatment on the expressions of c-MYC and cyclin-D1 proto-oncogenes

The expression levels of c-MYC increased 9.3 folds (p value= 0.003) in DMH-induced CRC mice (Group B) compared to the normal control group (Group A) as shown in Figure 2. DMSO also induced significant increase in the c-MYC expression (Group C) by 17.4 folds (p value= 0.011) compared to normal mice. Treatment with Sorafenib (Group D), CD8 T cell therapy (Group E) or their combination (Group F) induced significant decrease in c-MYC expression by 12.29 (p value= 0.004), 14.7 (p value= 0.003), and 16.49 (p value = 0.011) folds, respectively as compared to untreated CRC mice (Group C).

Similar results were obtained with cyclin-D1 gene expression. DMH and DMSO (Groups B and C) induced significant increase in cyclin-D1 expression levels by 16.3 (p value= 0.006) and 19.1 (p value= 0.003) folds respectively as compared to the normal group (Group A) (Figure 2). However, Sorafenib and/or CD8 T cell therapy (Groups D, E, and F) significantly decreased the expression levels of cyclin-D1 to 18.6, 18.1, and

ISSN: 03875547
Volume 44, Issue 05, October, 2021

18.7 folds (p values= 0.0001 each), respectively as compared to untreated CRC mice control (Group C).

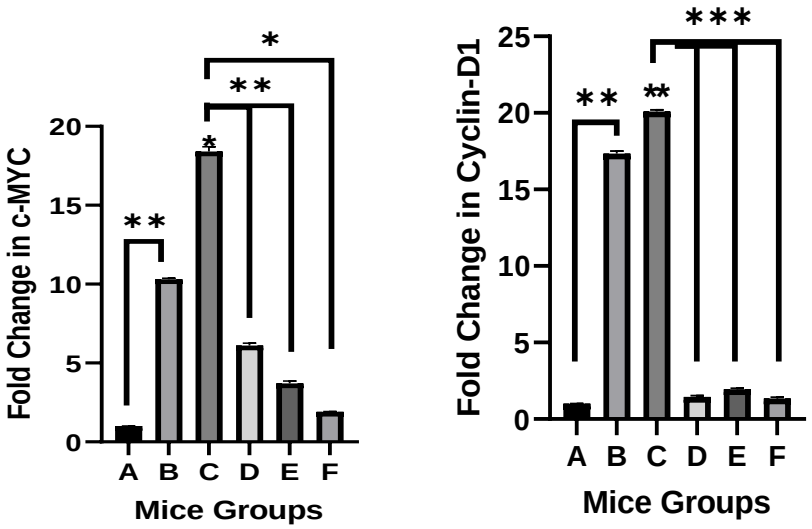


Figure 2. Effect of CD8 T cell therapy in presence or absence of Sorafenib on the expression levels of proto-oncogenes c-MYC and cyclin-D1 in colon tissues of all experimental mice groups. Expression levels of treated and control groups were normalized to their respective GAPDH. Fold expression was determined relative to the control. All bars represent mean of three determinations ± SD. (*), (**), and (***) on bars and on lines drawn upwards, that represent inter-categorical statistical significance, correspond to p<0.05, <0.01, and <0.001, respectively.

3.1.3 Effect of Sorafenib and CD8 T cell treatment on the expression levels of caspase-9 and CDC-4 tumor suppressor genes

No significant effect on the expression of caspase-9 was observed among the different groups compared to control. DMH and DMSO induced insignificant decrease in caspase-9 expression in (Groups B and C) by 0.5 folds (p value= 0.126, and 0.170, respectively) compared to the normal group (Group A) (Figure 3). Sorafenib and/or CD8 T cell therapy (Groups D, E, and F) induced non-significant increase in the expression levels of caspase-9 by 0.7 (p value= 0.064), 0.15 (p value= 0.372), and 0.88 (p value= 0.051) folds, respectively, compared to their untreated control group (Group C).

The effect on CDC-4 expression is shown in Figure 3. DMH (Group B) and vehicle (Group C) treatment induced a significant increase in the expression levels of CDC-4 by 0.94 (p value= 0.010) and 0.59 (p value = 0.011) folds, respectively compared to the normal control (Group A). Upon comparing CRC mice treated with the Sorafenib and/or CD8 T cell therapy (Groups D, E and F) to untreated CRC mice (Group C), a significant increase in CDC-4 expression levels by 2.53 (p value= 0.0001), 2.26 (p value=0.014), and 4.6 (p value= 0.020) folds, respectively was obtained.

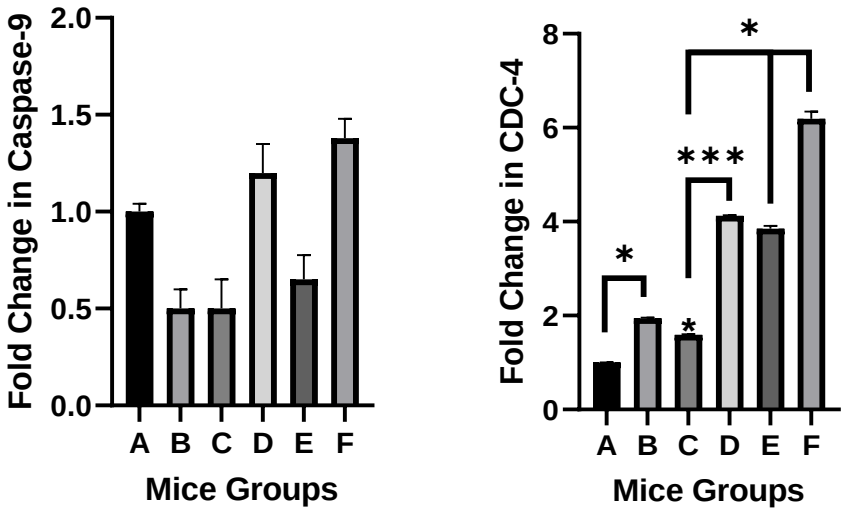
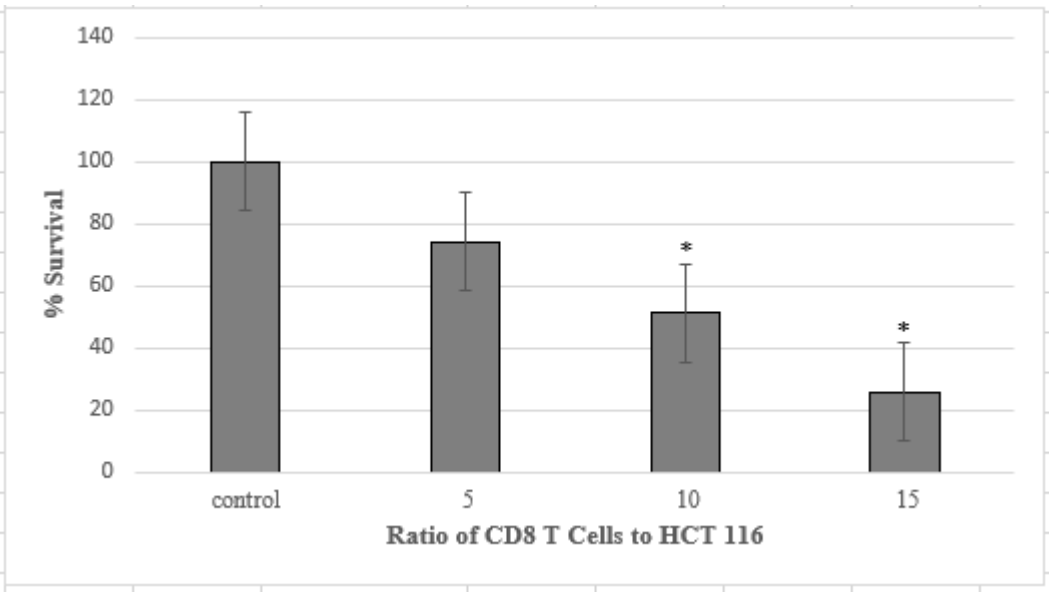


Figure 3. Effect of CD8 T cell therapy in presence or absence of Sorafenib on the expression levels of tumor suppressor genes (caspase-9 and CDC-4) in colon tissues of treated mice. Expression levels of treated and control groups were normalized to their respective GAPDH. Fold expression was determined relative to the control. All bars represent mean of three determinations ± SD. (*) and (***) on bars and on lines drawn upwards, that represent inter-categorical statistical significance, correspond to $p<0.05$ and <0.001, respectively.

II. In vitro

3.2 CD8 T cell Treatment against colon cancer cell lines

The CD8 T cells were co-cultured with colon cancer cell lines, HCT 116 and HCT 116 p53-/-, in three ratios 5:1, 10:1 and 15:1 CD8 to colon cancer cells. In both cell lines, the IC50 of CD8 T cells was significantly achieved at a ratio of 10:1 CD8 T cells to colon cancer cells (p value= 0.038 and 0.006 in HCT 116 and HCT 116 p53-/-, respectively) (Figure 4). The 5:1 CD8 T cell to colon cancer cells ratio resulted in survival rate of 74.04% in HCT 116 cell lines (p value= 0.09) and 78.69% in HCT 116 p53-/- (p value= 0.06). Moreover, the 15:1 ratio showed a survival rate of 25.7% among HCT 116 cells (p value= 0.029) and 20.11% among HCT 116 p53-/- cells (p value= 0.006).



ISSN: 03875547
Volume 44, Issue 05, October, 2021

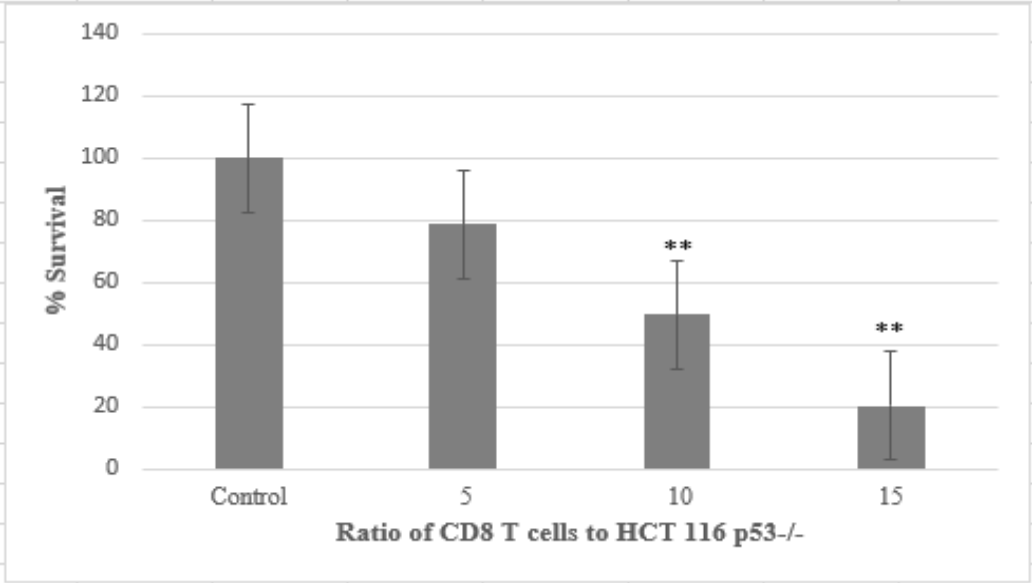


Figure 4. The killing capacity of different concentrations of CD8 T cells against HCT 116 and HCT 116 p53-/- colon cancer cells using MTT assay. All bars represent mean of three determinations ± SD. (*) and (**) on bars and on lines drawn upwards, that represent inter-categorical statistical significance, correspond to $p<0.05$ and <0.01, respectively.

3.3 Sorafenib Treatment against colon cancer cell lines

The ability of different concentrations of Sorafenib to kill colon cancer cells (HCT 116 and HCT 116 p53-/-) was assessed in vitro. Sorafenib concentration of 15 µM was able to reduce the survival of HCT 116 cell lines by 14.46% (p value= 0.018) (Figure 5). Similarly, Sorafenib concentrations of 25, 35, 50 and 75 µM significantly reduced the survival of HCT 116 cells by 27.04% (p value= 0.015), 34.33% (p value= 0.0006), 51.05% (p value= 0.003), and 59.86% (p value= 0.0004), respectively as compared to control cells. Therefore, the IC50 of Sorafenib against HCT 116 cell lines was 50 µM.

Similar results were observed in HCT 116 p53-/- whereby Sorafenib concentrations of 15, 25, 35, 50 and 75 µM were able to decrease the survival rate of HCT 116 p53-/- cells by 15.42% (p value= 0.18), 22.31% (p value= 0.07), 43.3% (p value= 0.009), 64% (p value= 0.001), and 73.5% (p value= 0.001), respectively as compared to control cells (Figure 5). The IC50 of Sorafenib against HCT 116 p53-/- cancer cell lines was 35 µM.

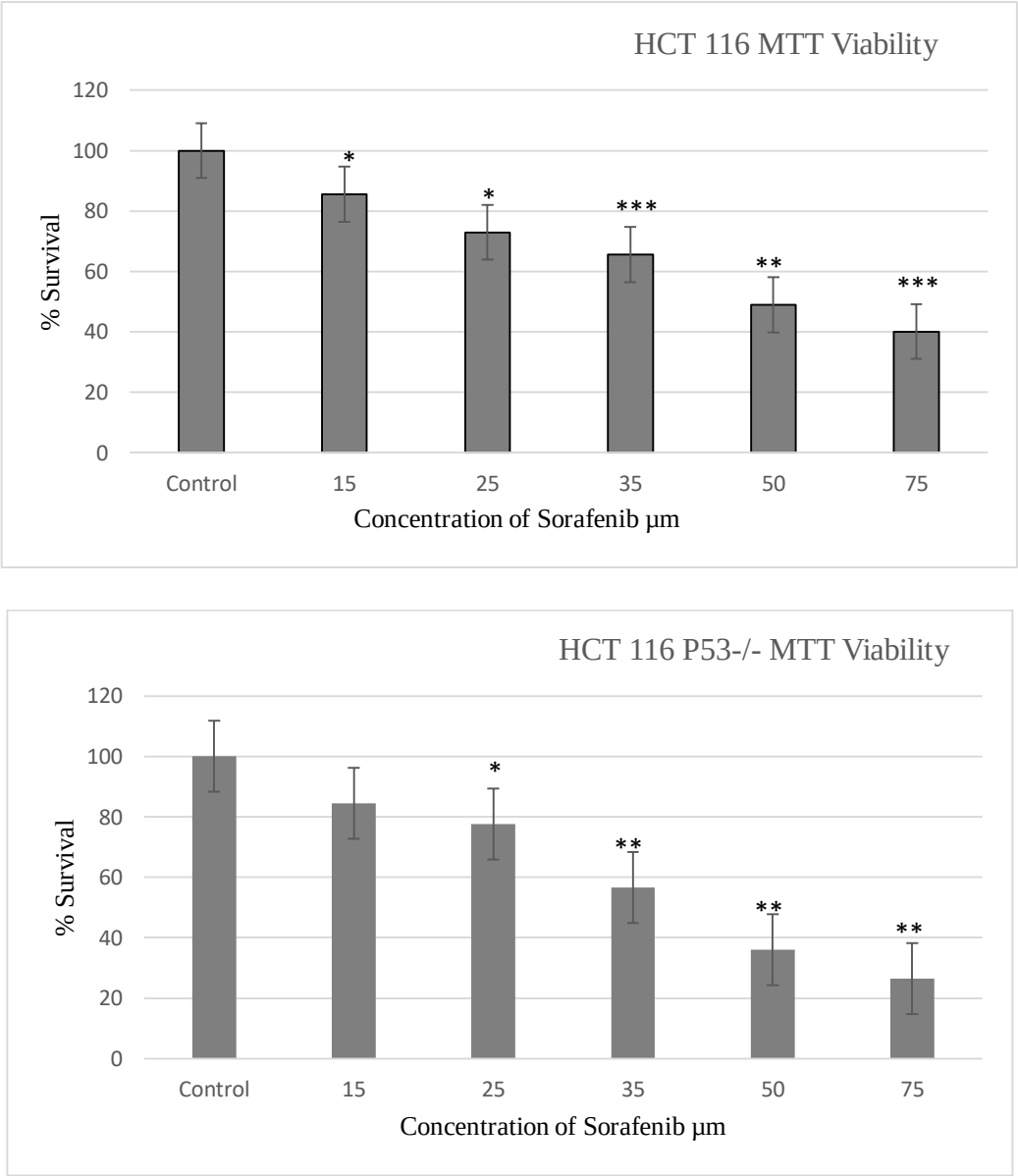


Figure 5. The killing capacity of different concentrations of Sorafenib against HCT 116 and HCT 116 p53-/- colon cancer cell lines using MTT assay. All bars represent mean of three determinations ± SD. (*), (**), and (***) on bars and on lines drawn upwards, that represent inter-categorical statistical significance, correspond to $p<0.05$, <0.01, and <0.001, respectively.

4. Discussion

Colon cancer progression occurs as a result of multiple mutations at the level of tumor-suppressor genes and oncogenes [17]. The platelet-derived growth factor/ platelet-derived growth factor receptor (PDGF/ PDGFR) pathway is a significant signaling pathway involved in tumor angiogenesis using surrounding vasculatures, drug resistance, and metastasis [18]. Two tyrosine kinase receptors, PDGFR alpha (PDGFRα) and PDGFR beta (PDGFRβ), and five ligands (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD) are involved in PDGF/ PDGFR angiogenic signaling pathway [19]. Upon binding of PDGF ligands to PDGF tyrosine kinase receptors; the latter will become phosphorylated. The phosphorylation of PDGFR will result in: a. the activation of various pathways (RAS/RAF/MEK/ERK pathway and PI3K/AKT/mTOR pathway) that lead to the transcription of c-MYC and cyclin-D1 involved in cell cycle activation and cancer proliferation in addition to the inhibition of apoptotic pathways involving caspase-9, caspase-3 and CDC-4

[20]. In this study, we analyzed the effect of Sorafenib, CD8 therapy and their combination on the expression of these genes. The role of proto-oncogene c-MYC in cell cycle control, apoptosis, DNA metabolism and dynamics, energy metabolism and macromolecular synthesis and its up-regulation in colon cancer have been investigated in several studies [21]. Cyclin-D1 is also a proto-oncogene known to be over-expressed in colon cancer cells resulting in an unchecked cell cycle progression, a high proliferation rate, and ultimately a more malignant phenotype [22]. Caspase-9 is a tumor suppressor gene known to induce apoptosis of colon cancer cells [23]. Caspase-9 is deregulated in colon cancer as an escape mode followed by cancer cells to escape apoptosis [8]. CDC-4 is another tumor suppressor gene known to be down-regulated in colon cancer cell progression [2]. Since PDGF signaling can also lead to an increase in interstitial fluid pressure (IFP) in most solid tumors, several chemotherapeutic treatments included the inhibition of PDGF signaling to increase the efficacy of cancer elimination/ inhibition [5]. A study conducted by used the tyrosine kinase inhibitor, Imatinib, to inhibit PDGF/PDGFR pathway, the caspase-9 pathway was then activated enhancing gastric cancer cells' apoptosis in vitro [20]. Immunotherapy is one of the advanced anticancer treatments nowadays after surgery and chemotherapy [11]. An in vitro study on HCT116 and SW480 cells suggested that the activation and generation of 90K-specific cytotoxic T lymphocytes using 90K peptide-pulsed dendritic cells could be used as an immunotherapeutic treatment, inhibiting colon cancer [24]. Therefore, in this work we aimed to study the effect of Adoptive T cell therapy (CD8 T cell) alone and in combination with chemotherapy on PDGF/PDGFR signaling pathway and on the expression of oncogenes and tumor suppressor genes (CDC-4, c-MYC, caspase-9 and cyclin-D1) in DMH-induced colon cancer in Balb/c mice.

Several studies have identified several tyrosine kinase receptors as targets for Sorafenib in chemotherapeutic cancer treatment, such as PDGFR [25]. Our study results were consistent with previous studies whereby the expression levels of PDGF-beta, PDGF-alpha receptor and PDGF-beta receptor were reduced in Sorafenib treated group. However, better results were obtained when Sorafenib was combined with CD8 T cells in comparison to either treatment alone. This is also suggested in another study where PDGFR kinase inhibitor ST1571 have resulted in apoptosis, inhibited tumor growth and prolonged survival in orthotopic murine models of ovarian carcinoma [26]. Moreover, it was also mentioned that specific CD8+ T cells are able to provide a steadier and stable phase of protection to patients with active or at risk of recurrent solid tumor [26]. To confirm CRC induction in our study, histological analysis of colonic tissues were performed. DMH induced structural abnormalities in the Lieberkühn glands and goblet cells depletion. Severe leukocyte infiltration in the lamina propria, and neoplastic cells of gland-like structures called lymphoid follicles accompanying cystic dilation were observed. Upon treatment of CRC mice with either Sorafenib, CD8 T cells or their combination restored the normal architecture of the colon tissue (results published in an accepted manuscript). In our study, mice receiving DMH (Group B) and vehicle/DMH (Group C) showed significant upregulations in the expression levels of c-MYC and cyclin-D1 in addition to downregulations in the expression levels of caspase-9 and CDC-4 compared to normal mice (Group A). Our results are consistent with other studies inregards to cyclin-D1, CDC-4, c-MYC and caspase-9 expression levels in untreated CRC mice versus healthy Balb/c mice. Several anticancer therapies targeted these genes to inhibit or slow down the progression of colon cancer cells [27].

Our findings also showed that the administration of Sorafenib and CD8 T cell induced a significant decrease in the expression levels of c-MYC. However, the combination of both Sorafenib and CD8 T cell treatment was more potent and significant in reducing the expression of c-MYC. Several studies were consistent with the reduced expression of c-MYC upon chemotherapeutic treatment in different types of cancer, especially in hepatocellular carcinoma [28]. Moreover, an in vivo study showed that mice immunized with human c- MYC protein and c-MYC derived non-homologous peptides obtained a c-MYC-

specific CD4+ and CD8+ T-cell response suggesting that oncogenic c-MYC can be targeted with specific T-cells [29]. In vivo studies on the antitumor activity of Sorafenib in patient derived Hepatocellular carcinoma (HCC) xenografts revealed that Sorafenib treatment moderately decreased the expression levels of cyclin-D1 [30]. This is consistent with our results where significant decrease in cyclin-D1 expression was elicited in Sorafenib treated mice and in mice treated with both Sorafenib and CD8 T cells. CD8 T cell treatment was also able to give results similar to Sorafenib in terms of its effect cyclin-D1 expression. These results confirm the study performed on the effect of CD8 T cells on cyclin-D1 in Mantle cell lymphoma where cyclin-D1 was considered a potentially important antigen for immunotherapy of MCL [31]. It is also obvious from our obtained findings that the expression of caspase-9 was significantly decreased in DMH mice and those administered DMSO. However, caspase-9 expression was restored upon treating the mice with chemotherapeutic drug (Sorafenib) and the combined treatment of both CD8 T cells and Sorafenib. This increase in the expression of caspase-9 was also noticed among CRC mice treated with CD8 T cell alone. This result is in compliance with other studies confirming the role of Sorafenib in enhancing tumor cell apoptosis by activating caspase-dependent pathways [32].

CDC-4 expression was shown to be slightly increased in the untreated CRC mice group as compared to normal group. Vehicle administration (Group C) induced a decrease in the level of CDC-4 as compared to the DMH group (Group B). Sorafenib treatment resulted in further increase in the expression of CDC-4. This is consistent with various studies where Sorafenib increases the expression levels of CDC-4 [33]. Although the effect of CD8 T cells on CDC-4 was not advanced in previous studies, CD8 T cell treatment alone or in combination with Sorafenib was also able to increase the expression of CDC-4 as shown in our study. Interestingly, our study is the first to demonstrate the in vivo effect of CD8 T cells alone or in combination with Sorafenib on the expression of oncogenes, tumor suppressor genes, and the PDGF/PDGFR in DMH- induced colon cancer in Balb/c mice. We also studied the effect of CD8 T cells and Sorafenib on colon cancer cell lines (HCT 116 and HCT 116 p53-/-) in vitro using MTT assay. Dendritic cells were used to activate CD8 T cells against cancer cell lysates. The primary function of dendritic cells 1 (DC1) is to enhance immunity via the stimulation of CTLs and hence, the initiation of Th1-immune response. In the same study, dendritic cells were activated against SW480 and SW620 colon cancer cell lines in vitro [34]. Activated CTLs kill tumor cells by direct cytotoxicity or by activation of naïve CTLs and initiation of Th1type tumor immunoreactions [34]. Also proved that mature dendritic cells loaded using cancer cell lysates induce CTL activation, and that activated CTLs had cytotoxic effects against cancer cells. This was referred to as dendritic cells vaccines i.e. the most powerful antigen presenting cells that may aid in CD8 T cell activation and therefore, resulting in the success of adoptive T cell therapy [34].

Several studies have reported the role of Sorafenib in inhibiting proliferation and activating apoptosis in multiple cell lines such as colon, pancreas, melanoma, leukemia, thyroid, malignant glioma, lung, breast, and cholangiocarcinoma cell lines [35]. In one of the studies, it was reported that Sorafenib treatment might overcome the resistance induced by EGFR to Tyrosine Kinase Inhibitors in colon cancer cell lines [36]. Moreover, it was reported that Sorafenib blocks B-RAF in addition to being able to inhibit cancer cell lines expressing mutated K-RAS genes [36]. Although the activity of Sorafenib was reported in these multiple studies, we tended to detect the relative sensitivities using a simple assay (MTT) in 2 colon cancer cell lines (HCT 116 and HCT 116 p53-/-) in order to detect growth inhibition and compare it to that of CD8 T cells. In our study, the IC50 of Sorafenib against HCT 116 p53-/- and HCT 116 cancer cell lines was 35 µM and 50 µM, respectively. The IC50 of Sorafenib detected in this study is somehow higher than that reported in other studies against HCT116 (3 µM) (Mazard 2013) and were somehow different than other studies reporting IC50 of Sorafenib on HCT 116. The ratios of CTLs to colon cancer cell lines that was used in

MTT assay was in reference to the ratio reported by (i.e. 10:1) [34]. Our results showed that the IC50 of CD8 T cells in both cell lines was 10:1 CD8 T cells to colon cancer cell lines ratio. It was hard to study the effect of a combination therapy of both Sorafenib and CD8 T cells in vitro since the cell lines did not survive any concentration and therefore, monitoring growth was not applicable (results not shown). To our knowledge, there are no previous data reporting the killing capacity of CD8 T cells on HCT116 (wild and mutant) colon cancer cell lines.

5. Conclusion

In conclusion, due to the side effects caused by various chemotherapeutic drugs including Sorafenib, CD8 T cell therapy (also called Adoptive T cell therapy) can be used as a substituent or an adjunct to chemotherapy in colon cancer treatment. Further studies are required especially that our sample size was small relatively. However, our data may open up future work for using Adoptive immunotherapy in colon cancer treatment targeting the PDGF/PDGFR, oncogenes (c-MYC and cyclin-D1) and tumor suppressor genes (caspase-9 and CDC-4).

6. Acknowledgement

The authors would like to thank Dr. Hani Tamim for assisting in statistical analysis.

7. References

[1] Spallanzani A, Gelsomino F, Caputo F, Santini C, Andrikou K, Orsi G, et al. Immunotherapy in the treatment of colorectal cancer: a new kid on the block. Journal of Cancer Metastasis and Treatment 2018; 4(6):28.

[2] Korphaisarn K, Pongpaibul A, Roothumnong E, Pongsuktavorn K, Thamlikitkul L, Anekpuritanang T, et al. High Frequency of KRAS Codon 146 and FBXW7 Mutations in Thai Patients with Stage II- III Colon Cancer. Asian Pac J Cancer Prev 2019; 20(8):2319-2326.

[3] Zhong L, Zhang X, Covasa M. Emerging roles of lactic acid bacteria in protection against colorectal cancer. World J Gastroenterol 2014; 20(24):7878-7886.

[4] Zhao M, Yu Z, Li Z, Tang J, Lai X, Liu L. Expression of angiogenic growth factors VEGF, bFGF and ANG1 in colon cancer after bevacizumab treatment in vitro: A potential self-regulating mechanism. Oncol Rep 2017; 37(1):601-607.

[5] Liu KW, Hu B, Cheng SY. Platelet-derived growth factor signaling in human malignancies. Chin J Cancer 2011; 30(9):581-584.

[6] Au W-C, Zhang T, Mishra PK, Eisenstatt JR, Walker RL, Ocampo J, et al. Skp, Cullin, F-box (SCF)-Met30 and SCF-Cdc4-Mediated proteolysis of CENP-A prevents mislocalization of CENP-A for chromosomal stability in budding yeast. PLoS genetics 2020; 16(2):e1008597.

[7] Fu Y,Yang G, Xue P, Guo L, Yin Y, Ye Z, et al. Dasatinib reduces 5-Fu-triggered apoptosis in colon carcinoma by directly modulating Src-dependent caspase-9 phosphorylation. Cell death discovery 2018; 4:61.

[8] Li P, Zhou L, Zhao T, Liu X, Zhang P, Liu Y, et al. Caspase-9: structure, mechanisms and clinical application. Oncotarget 2017; 8(14):23996-24008.

[9] Ren L, Zhou T, Wang Y, Wu Y, Xu H, Liu J, et al. RNF8 induces β-catenin-mediated c-Myc expression and promotes colon cancer proliferation. International journal of biological sciences 2020; 16(12):2051-2062.

[10] Pysz MA, Hao F, Hizli AA, Lum MA, Swetzig WM, Black AR, et al. Differential regulation of cyclin D1 expression by protein kinase C α and ϵ signaling in intestinal epithelial cells. The Journal of biological chemistry 2014; 289(32):22268-22283.

[11] Olson B, Li Y, Lin Y, Liu ET, Patnaik A. Mouse Models for Cancer Immunotherapy Research. Cancer Discovery 2018; 8(11):1358.

[12] Yang Y. Cancer immunotherapy: harnessing the immune system to battle cancer. The Journal of Clinical Investigation 2015; 125(9):3335-3337.

[13] Boghossian S, Robinson S, Von Delwig A, Manas D, White S. Immunotherapy for treating metastatic colorectal cancer. Surgical oncology 2012; 21(2):67-77.

[14] Turcotte S, Gros A, Tran E, Lee C-CR, Wunderlich JR, Robbins PF, et al. Tumor-reactive CD8+ T cells in metastatic gastrointestinal cancer refractory to chemotherapy. Clinical Cancer Research 2014; 20(2):331-343.

[15] Wang ZX, Cao JX, Liu ZP, Cui YX, Li CY, Li D, et al. Combination of chemotherapy and immunotherapy for colon cancer in China: a meta-analysis. World J Gastroenterol 2014; 20(4):1095- 1106.

[16] Kacan T, Nayir E, Altun A, Kilickap S, Babacan NA, Ataseven H, et al. Antitumor activity of sorafenib on colorectal cancer. Journal of Oncological Sciences 2016; 2(2-3):53-57.

[17] Armaghany T, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. Gastrointestinal cancer research: GCR 2012; 5(1):19.

[18] Liu K-W, Hu B, Cheng S-Y. Platelet-derived growth factor signaling in human malignancies. Chinese journal of cancer 2011; 30(9):581.

[19] Jansson S, Aaltonen K, Bendahl P-O, Falck A-K, Karlsson M, Pietras K, et al. The PDGF pathway in breast cancer is linked to tumour aggressiveness, triple-negative subtype and early recurrence. Breast cancer research and treatment 2018; 169(2):231-241.

[20] Kim JL, Lee DH, Jeong S, Kim BR, Na YJ, Park SH, et al. Imatinib-induced apoptosis of gastric cancer cells is mediated by endoplasmic reticulum stress. Oncology Reports 2019; 41(3):1616-1626.

[21] Venkateswaran N, Lafita-Navarro MC, Hao Y-H, Kilgore JA, Perez-Castro L, Braverman J, et al. MYC promotes tryptophan uptake and metabolism by the kynurenine pathway in colon cancer. Genes & development 2019; 33(17-18):1236-1251.

[22] Ripple MJ, Parker Struckhoff A, Trillo-Tinoco J, Li L, Margolin DA, McGoey R, et al. Activation of c-Myc and Cyclin D1 by JCV T-Antigen and β-Catenin in Colon Cancer. PLOS ONE 2014; 9(9):e106257.

[23] Saxena A, Fayad R, Kaur K, Truman S, Greer J, Carson JA, et al. Dietary selenium protects adiponectin knockout mice against chronic inflammation induced colon cancer. Cancer biology & therapy 2017; 18(4):257-267.

[24] Lee JH, Park M-S, Hwang J-E, Cho S-H, Bae W-K, Shim H-J, et al. Dendritic cell-based immunotherapy for colon cancer using an HLA-A* 0201-restricted cytotoxic T-lymphocyte epitope from tumor-associated antigen 90K. Cellular & molecular immunology 2013; 10(3):275-282.

[25] Zheng M, Xu H, Liao X-H, Chen CP, Zhang AL, Lu W, et al. Inhibition of the prolyl isomerase Pin1 enhances the ability of sorafenib to induce cell death and inhibit tumor growth in hepatocellular carcinoma. Oncotarget 2017; 8(18):29771.

[26] Komita H, Zhao X, Taylor JL, Sparvero LJ, Amoscato AA, Alber S, et al. CD8+ T-cell responses against hemoglobin-β prevent solid tumor growth. Cancer research 2008; 68(19):8076-8084.

[27] Lee C-W, Chen H-J, Xie G-R, Shih C-K. Djulis (Chenopodium formosanum) prevents colon carcinogenesis via regulating antioxidative and apoptotic pathways in rats. Nutrients 2019; 11(9):2168.

[28] Liu Y, Lou G, Norton JT, Wang C, Kandela I, Tang S, et al. 6-Methoxyethylamino-numonafide inhibits hepatocellular carcinoma xenograft growth as a single agent and in combination with sorafenib. The FASEB Journal 2017; 31(12):5453-5465.

[29] Helm F, Kammertoens T, Lehmann FM, Wilke A, Bruns H, Mautner J, et al. Targeting c-MYC with T-cells. PloS one 2013; 8(10):e77375.

[30] Wu W-D, Chen P-S, Omar HA, Arafa E-SA, Pan H-W, Jeng J, et al. Antrodia cinnamomea boosts the anti-tumor activity of sorafenib in xenograft models of human hepatocellular carcinoma. Scientific reports 2018; 8(1):1-13.

[31] Wang M, Sun L, Qian J, Han X, Zhang L, Lin P, et al. Cyclin D1 as a universally expressed mantle cell lymphoma-associated tumor antigen for immunotherapy. Leukemia 2009; 23(7):1320-1328.

[32] Hamed HA, Tavallai S, Grant S, Poklepovic A, Dent P. Sorafenib/regorafenib and lapatinib interact to kill CNS tumor cells. Journal of cellular physiology 2015; 230(1):131-139.

[33] Sailo BL, Banik K, Girisa S, Bordoloi D, Fan L, Halim CE, et al. FBXW7 in cancer: What has been unraveled thus far? Cancers 2019; 11(2):246.

[34] Chen L, Meng D, Zhao L, Liu R, Bai P, Wang L, et al. Selective colorectal cancer cell lysates enhance the immune function of mature dendritic cells in vitro. Molecular medicine reports 2015; 11(3):1877- 1884.

[35] Plastaras JP, Kim S-H, Liu YY, Dicker DT, Dorsey JF, McDonough J, et al. Cell Cycle–Dependent and Schedule-Dependent Antitumor Effects of Sorafenib Combined with Radiation. Cancer Research 2007; 67(19):9443.

[36] Morgillo F, Martinelli E, Troiani T, Orditura M, De Vita F, Ciardiello F. Antitumor activity of

sorafenib in human cancer cell lines with acquired resistance to EGFR and VEGFR tyrosine kinase inhibitors. PLoS One 2011; 6(12):e28841.

[37] He J, Song Y, Li G, Xiao P, Liu Y, Xue Y, et al. Fbxw7 increases CCL2/7 in CX3CR1 hi macrophages to promote intestinal inflammation. The Journal of clinical investigation 2019; 129(9).

[38] Xu W, Guo G, Li J, Ding Z, Sheng J, Li J, et al. Activation of Bcl-2-caspase-9 apoptosis pathway in the testis of asthmatic mice. Plos one 2016; 11(3):e0149353.

[39] El Joumaa MM, Taleb RI, Rizk S, Borjac JM. Protective effect of Matricaria chamomilla extract against 1, 2-dimethylhydrazine-induced colorectal cancer in mice. Journal of Complementary and Integrative Medicine 2020; 1(ahead-of-print).

[40] Fan B, Ma L, Li Q, Wang L, Zhou J, Wu J. Correlation between platelet-derived growth factor signaling pathway and inflammation in desoxycorticosterone-induced salt-sensitive hypertensive rats with myocardial fibrosis. International Journal of Clinical and Experimental Pathology 2013; 6(11):2468.

[41] Basma H, Ghayad SE, Rammal G, Mancinelli A, Harajly M, Ghamloush F, et al. The synthetic retinoid ST1926 as a novel therapeutic agent in rhabdomyosarcoma. International Journal of Cancer 2016; 138(6):1528-1537.